Studies on the development and reproductive biology of Echinostomaliei (Digenea : Echinostomatidae) in the mouse host

Balogun, M. A

Studies on the Development and Reproductive Biology of *Echinostoma liei* (Digenea: Echinostomatidae) in the mouse host.

b y

Monsurudeen Adekoyejo Balogun B.Sc. (Hons).

A thesis submitted for the degree of Doctor of Philosophy in the Faculty of Science of The University of London

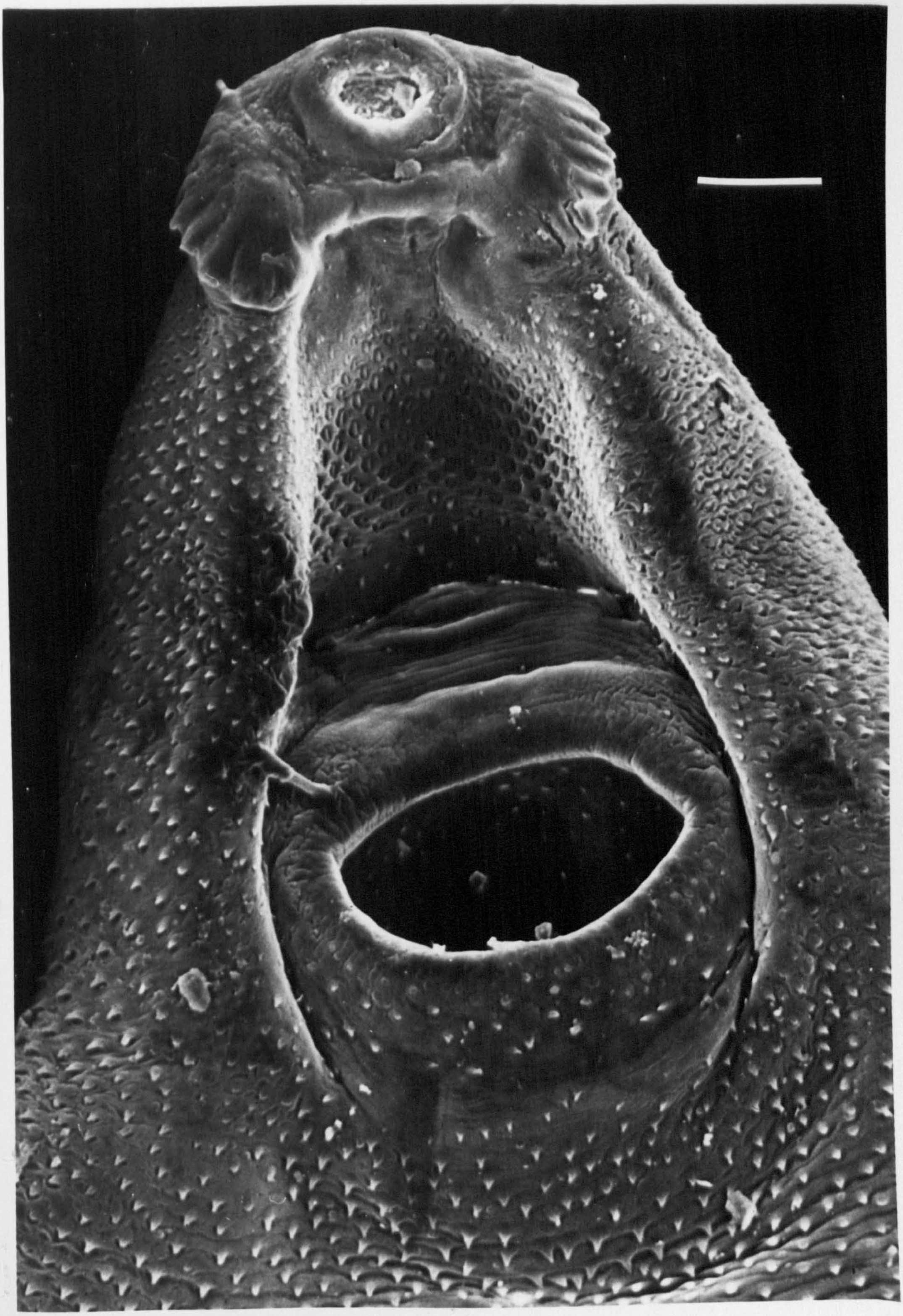
1991

King's College London
University of London
Division of Biosphere Sciences

Frontispiece (facing page)

Scanning electron micrograph of the anterior region of an adult *Echinostoma liei*

Scale bar = 100 μm



Abstract

The 37-spined echinostomatid digenean, *Echinostoma liei* (probably synonymous with *Echinostoma caproni*, Richard 1964) has been maintained in the laboratory utilizing experimental passage through *Biomphalaria glabrata* (as first and second intermediate hosts) and male Swiss T.O. mice (as final hosts). Using this system, several aspects of the worm's biology in the mouse host have been investigated.

Preliminary studies involved quantitative investigation of the somatic growth and development of the worms in their final hosts. Detailed findings were produced concerning the pattern of development of the vitellaria along with the ultrastructural observations on the process of spermatogenesis. Experimental infection studies involved the analysis of the distribution and gut microhabitat utilization of *E. liei* in the intestines of Swiss T.O. mice and the ontogenetic migratory behaviour asssociated with such infections. Density-dependent effects on worms of *E. liei* in the intestines of mice were examined. Alongside this, the pathogenesis associated with such infections, initially exposed to metacercarial cyst doses of a range of sizes were observed.

Aspects of the worm's neurobiology were investigated using indirect immunolabelling techniques which identified a sub-population of immunoreactive sub-tegumentary cells in developing

worms. The implication of this and its importance in the host/parasite interplay are discussed.

In a separate group of experimental infections it was established that monometacercarial cyst infections in mice are capable of producing viable eggs that can be used to successfully infect *B. glabrata*. This finding has been experimentally exploited in a long-term experiment which compared parasite reproductive success in a number of ways in separate selfing and outbreeding lines. After four generations the reproductive success of the selfing line collapsed. Ultrastructural observations revealed that in such self-inseminated worms, the process of spermatogenesis was halted. The biological importance of this finding for the reproduction of *E. liei* is discussed.

Acknowledgements

I would like to thank Dr. P.J. Whitfield, my supervisor, for his help, guidance and continual encouragement during the course of this study.

I would also like to thank Alan Howard, Andrew Langridge, Alan Maple and Harry Edge for their advice and technical assistance.

I am also very grateful to Dr. M.C. Thorndyke and Jennifer Riddell for their help and loan of facilities and equipment in the Biology Department of Royal Holloway and Bedford New College.

It is also a pleasure to thank Dr. V.R. Southgate for his help and technical assistance for work conducted in the Zoology Department of the British Natural History Museum.

Finally I would like to show my appreciation to Dr. A. M. McCarthy, Dr. N. A. Evans, Dr. N. O. Christensen and Professor B. Fried, for some interesting discussions on echinostomes.

Contents

Chapter 6 Parasite population density-related effects in *Echinostoma liei* infections in mice and the pathogenesis associated with infection

List of Figures

List of Tables

CHAPTER 1

INTRODUCTION

ECHINOSTOMA LIEI (JEYARASASINGAM, HEYNEMAN, LIM AND MANSOUR, 1972), AN ECHINOSTOMATID DIGENEAN

1.1 Introduction

Echinostoma liei (Jeyarasasingam, Heyneman, Lim and Mansour, 1972) is a member of the family Echinostomatidae, a group of digeneans characterised by the presence of a number of collar spines which surround the oral sucker. This morphological feature is found in the cercariae, metacercarial and adult stages of the life cycle and has been used as a key feature in the classification of members of this taxon. *E. liei* belongs to a small group within the genus *Echinostoma* which possess 37 of these collar spines. Jeyarasasingam and his co-workers pointed out that *E. liei* was the first African member of this 37-spined group to be described in detail.

The life cycle of the digenean parasite *E. liei* was first described by Jeyarasasingam, *et al.* (1972) after these workers had observed pulmonate snails, *Biomphalaria alexandrina*, collected from irrigation canals in the Nile Delta near Cairo shedding echinostome cercariae. Jeyarasasingam *et al.* (1972) subsequently named the parasite after they had described a 21 day-old adult of this species which they had recovered from hamsters fed metacercarial cysts removed from the pericardial sac of *Biomphalaria glabrata*. They found that naturally infected *B. alexandrina* snails from Egypt shed echinostome cercariae which were able to penetrate laboratory-reared *B. glabrata*. *B. alexandrina*, they noted, served both as the first and second intermediate host under natural conditions. Experimental work revealed that both chicks and hamsters were suitable definitive hosts for the adult worms of this echinostome.

Further work carried out by Jeyarasasingam and his co-workers demonstrated that *E. liei* was able to utilize a broad spectrum of both avian and mammalian definitive hosts both naturally and under experimental conditions. These authors showed that the natural hosts included the Egyptian giant shrew (*Crocidura olivieri*), rats (*Rattus rattus*) and probably other rodents and aquatic birds, while under experimental conditions they demonstrated that the parasite could be recovered from pigeons (*Columba livia*), finches (*Lonchura striata*), mice (*Mus musculus*) and hamsters (*Mesocricetus auratus*).

Kuris (1980a,b) and Kuris and Warren (1980) have referred to an Ethiopian strain of *E. liei* in their work on echinostomes which they indicated was the same parasite described by Jeyarasasingam *et al.* (1972). Moravec, Barus, Rysavy and Yousif (1974) observed echinostome cercariae shed from a naturally infected population of *B. alexandrina* collected from the same irrigation system and in the same area where Jeyarasasingam *et al.* (1972) identified *E. liei* but named this parasite *Echinostoma revolutum*. Jeyarasasingam *et al.*, (1972) suggested that *E. liei* differed from *Echinostoma revolutum* (Beaver, 1937) mainly in the different number and arrangement of the finfolds on the cercarial tail, the absence in the cercariae of paraoesophageal gland cells and the number of penetration gland outlets on the dorsal lip of the oral sucker. Moravec *et al.* (1974) explained (as did Lie and Basch, 1967 in their description of the Brazilian echinostome *Echinostoma paraensei*) that the number of penetration gland outlets is not necessarily a constant feature in

cercariae of the genus *Echinostoma*. They therefore concluded that there was no reason to consider the Egyptian echinostome to be a separate species from the European *E. revolutum*. Interestingly, these authors found that the morphology of all the developmental stages of the Egyptian *E. revolutum* were consistent with *E. liei* leading these authors to believe that *E. revolutum* and *E. liei* were conspecific.

Recent systematic studies on echinostomes carried out by Kanev (1985) have indicated that the taxonomic analysis of 37-spined echinostomes is in considerable disarray. With the knowledge obtained in his study using morphological, biological and ecological data, Kanev established a new taxonomic system for this group. In his scheme, important criteria for differentiating echinostomes included the species of the first intermediate hosts and the final hosts, the number and position of the penetration and paraoesophageal gland cell openings and starch gel analyses of parasite proteins. Kanev also took into account the geographical distribution of these echinostomes. With respect to the African echinostomes, according to Kanev *Echinostoma caproni* is the appropriate name for the echinostomes described under the names of *E. caproni* from Madagascar (Richard, 1964), *E. togoensis* from Togo (Jourdane and Kulo, 1981) and *E. liei* from Egypt and Ethiopia (Jeyarasasingam *et al.* 1972 and Kuris 1980a,b respectively) and *E. revolutum* from Egypt (Moravec *et al.* 1974). Kanev stressed that these echinostomes all had the same features as *E. caproni*, the life cycle of which was first decribed by Richard and Brygoo (1978).

Christensen, Fried and Kanev (1990) and Huffman and Fried (1990) in reviews of the taxonomy of the 37-collar spined *Echinostoma* have adopted this taxonomical system and have placed certain echinostomes in synonymy with one another according to the general stipulations made by Kanev (1985). In these reviews *E. caproni* (Richard, 1964) is regarded as the correct name for *E. liei* and the other African echinostomes, whilst *E. trivolvis* is regarded as the correct name for the North American *E. revolutum* described by Beaver (1937) and used in numerous studies by Fried and his co-workers between 1969 to 1988, while *E. revolutum* and *E. echinatum* are regarded as the correct names for 37-spined echinostomes from both Europe and Asia. A summary of the geographical location of the African echinostomes is provided in Table 1.1.

With respect to the 37-spined echinostomes of African origin, many of Kanev's conclusions have been consolidated by isoenzymatic analytical studies. An isoenzyme comparison between a natural population of *E. caproni* and two strains of *E. caproni* and *E. togoensis* maintained long term in the laboratory has suggested that *E. caproni* and *E. togoensis* are not different species but two geographical isolates of the same taxon implying that *E. togoensis* is a sub-species of *E. caproni* (Voltz, Richard and Pesson, 1987). Similarly, Voltz, Richard, Pesson and Jourdane, (1988) have shown, using isoenzyme analytical techniques, that the African strains of *E. liei, E. togoensis* and *E. caproni* and *E. sp.* (Cameroon) correspond to variants of the same species. These workers established

TABLE 1.1 The geographical location of the 37-spined African echinostomes considered synonyms of Echinostoma caproni

Name	Location	Natural first intermediate snail host	Author
E.caproni	Madagascar	B.pfeifferi	Richard (1964) Richard and Brygoo (1978)
E.liei	Egypt	B.alexandrina	Jeyarasasingam et al.(1972)
E.liei	Ethiopia	B.alexandrina	Jeyarasasingam et al.(1972) Kuris (1980a,b)
E.revolutum	Egypt	B.alexandrina	Moravec et al. (1974)
E.togoensis	Togo	B.pfeifferi	Jourdane and Kulo (1981)
E.sp.	Cameroon	not stated	Voltz, Richard,Pesson and Jourdane (1988)

successful interbreeding between these four putative species and obtained a second generation of hybrids. They commented that their results were in accord with the hypothesis of Kanev in which *E. liei* and *E. togoensis* were synonyms of *E. caproni*. Vasilev, Mikov, Kanev and Fried (1984) in a comparative electrophoretic study of the North American and European strains of *E. revolutum* indicated that the North American *E. revolutum* may not actually belong to the European species. Ross, Fried and Southgate (1989) later demonstrated, using an analysis of adult worm isoenzymes, that clear interspecific differences existed between adult *E. revolutum* (North America) and *E. liei* (Egypt).

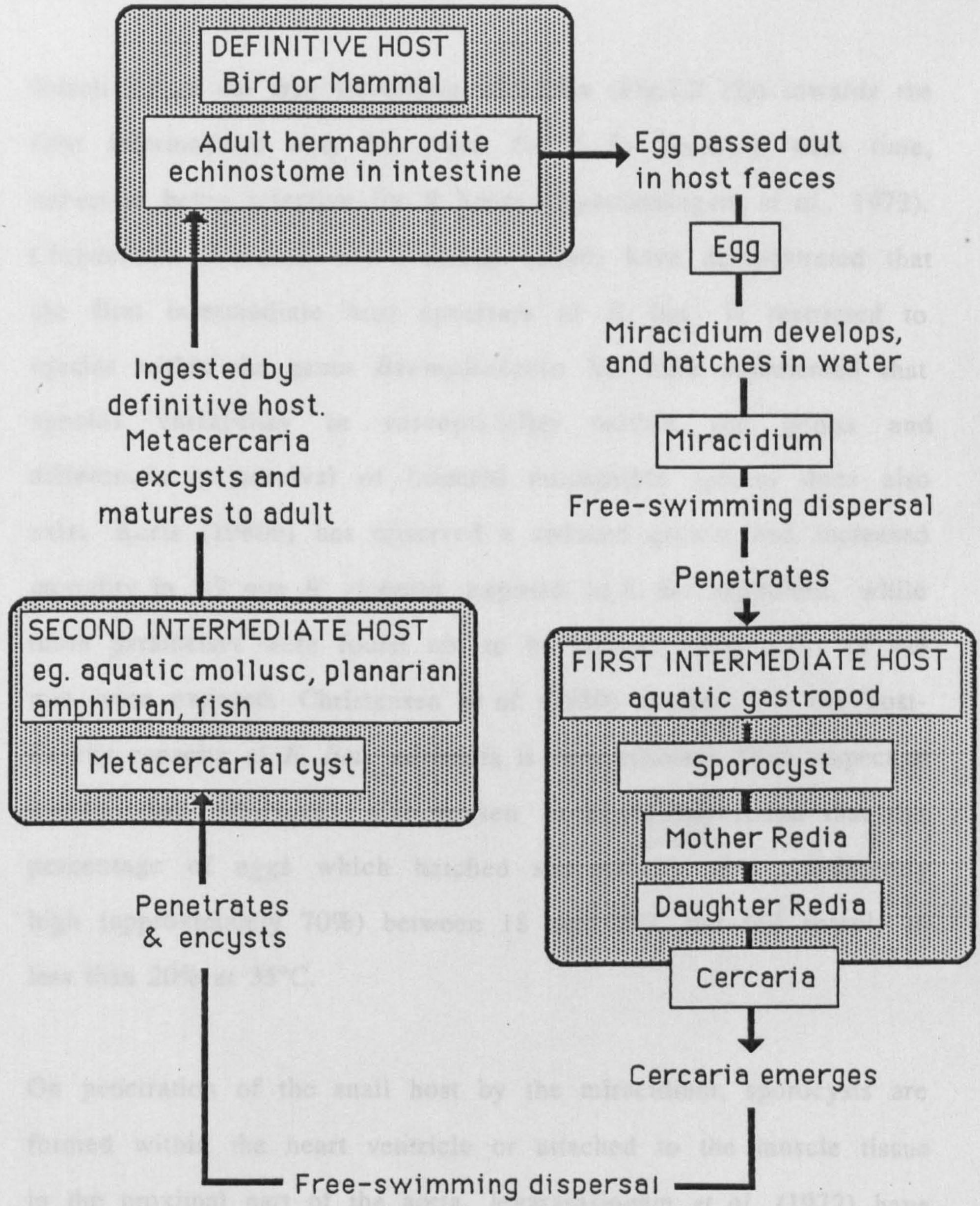
Given the highly confused state of the nomenclature of African 37-spined echinostomes which has been described above, careful thought has been given to the systematic terminology which will be used in this thesis. For reasons of clarity and conciseness the name *E. liei* has been retained here for the Egyptian-derived echinostome which has been utilized in all the experiments in this thesis. Where reference is made to other echinostomes the terminology used by the original authors has been conserved. This policy, however, in no way implies that the revision suggested by Kanev (1985) is not regarded as appropriate. Indeed it seems to bring order to an otherwise complex terminology. In reading this thesis, therefore, it must be remembered that references to forms such as *E. caproni*, *E. togoensis*, *E. revolutum* (from Africa) and *E. liei* almost certainly refer to isolates of a single species.

1.2 The life cycle of *E. liei*

The initial description of the developmental stages of *E. liei* was carried out by Jeyarasasingam *et al.* (1972). A detailed description of the maintenance of *E. liei* under laboratory conditions is provided in Chapter 2. The family Echinostomatidae includes numerous species of worms parasitic in many different kinds of vertebrate. A generalised summary of the echinostomatid life cycle is shown in Fig.1.1. Most species are characterised by a spiny outer tegumental covering and collar spines surrounding the oral sucker which have been used as aids in classifying species in this diverse family. *E. liei* is characterised by 37-collar spines with a pattern of (3+2) corner spines in each lappet, 6 laterals on each side and 15 dorsals in alternating rows (Jeyarasasingam *et al.*, 1972). The collar spine number and its arrangement seem to be the most reliable taxonomic character so far used in the taxonomic consideration of this group.

The hermaphroditic adult worms of *E. liei* which live attached to the intestinal wall of their vertebrate definitive host are characteristically dorso-ventrally flattened. Eggs are passed out of the uterus into the lumen of the intestine where they then leave the host via the faeces. Eggs are unembryonated when they are laid, oval in shape and have a yellowish-brown appearance (Fig.1.2 (1)). At one end of the egg is a clearly visible operculum. The unembryonated operculate eggs mature in distilled water at 26°C and are stimulated to hatch by light, releasing miracidia between 8

FIG 1.1 Diagram to show the generalized life cycle pattern of an echinostome digenean



and 12 days after release from adult worms (Jeyarasasingam *et al.* 1972; Kuris, 1980a,b).

Infectivity of the free swimming miracidia (Fig.1.2 (2)) towards the first intermediate host has been found to decrease with time, miracidia being infective for 8 hours (Jeyarasasingam *et al.*, 1972). Christensen, Frandsen and Roushdy (1980) have demonstrated that the first intermediate host spectrum of *E. liei* is restricted to species within the genus *Biomphalaria* but have commented that species variability in susceptibility within the genus and differences in survival of infected susceptible species does also exist. Kuris (1980b) has observed a reduced growth and increased mortality in 1-2 mm *B. glabrata* exposed to *E. liei* miracidia, while these parameters were found not to be altered when snails of 4-6 mm were exposed. Christensen *et al.* (1980) showed that the host-finding capacity of *E. liei* miracidia is very efficient. With respect to transmission efficiency Christensen *et al.* (1980) found that the percentage of eggs which hatched successfully was consistently high (approximately 70%) between 18 and 28°C but fell sharply to less than 20% at 35°C.

On penetration of the snail host by the miracidium, sporocysts are formed within the heart ventricle or attached to the muscle tissue in the proximal part of the aorta. Jeyarasasingam *et al.* (1972) have explained that the first generation (mother) rediae are released from the sporocysts from 6 days onwards post-miracidial exposure. By about day 10 onwards these first generation rediae migrate via

Fig. 1.2 (1-4) Larval forms of *E. liei*

(1) Egg of *E. liei* recovered from the faeces of an experimentally infected mouse
Scale bar= 20 μm

(2) Single miracidium of *E. liei* released from a fully embryonated egg
Scale bar= 50 μm

(3) A cercariae of *E. liei* released from a laboratory infected *B. glabrata* snail
Scale bar= 150 μm

(4) Metacercarial cyst of *E. liei* dissected from the pericardium of a laboratory infected *B. glabrata* snail
Scale bar= 50 μm

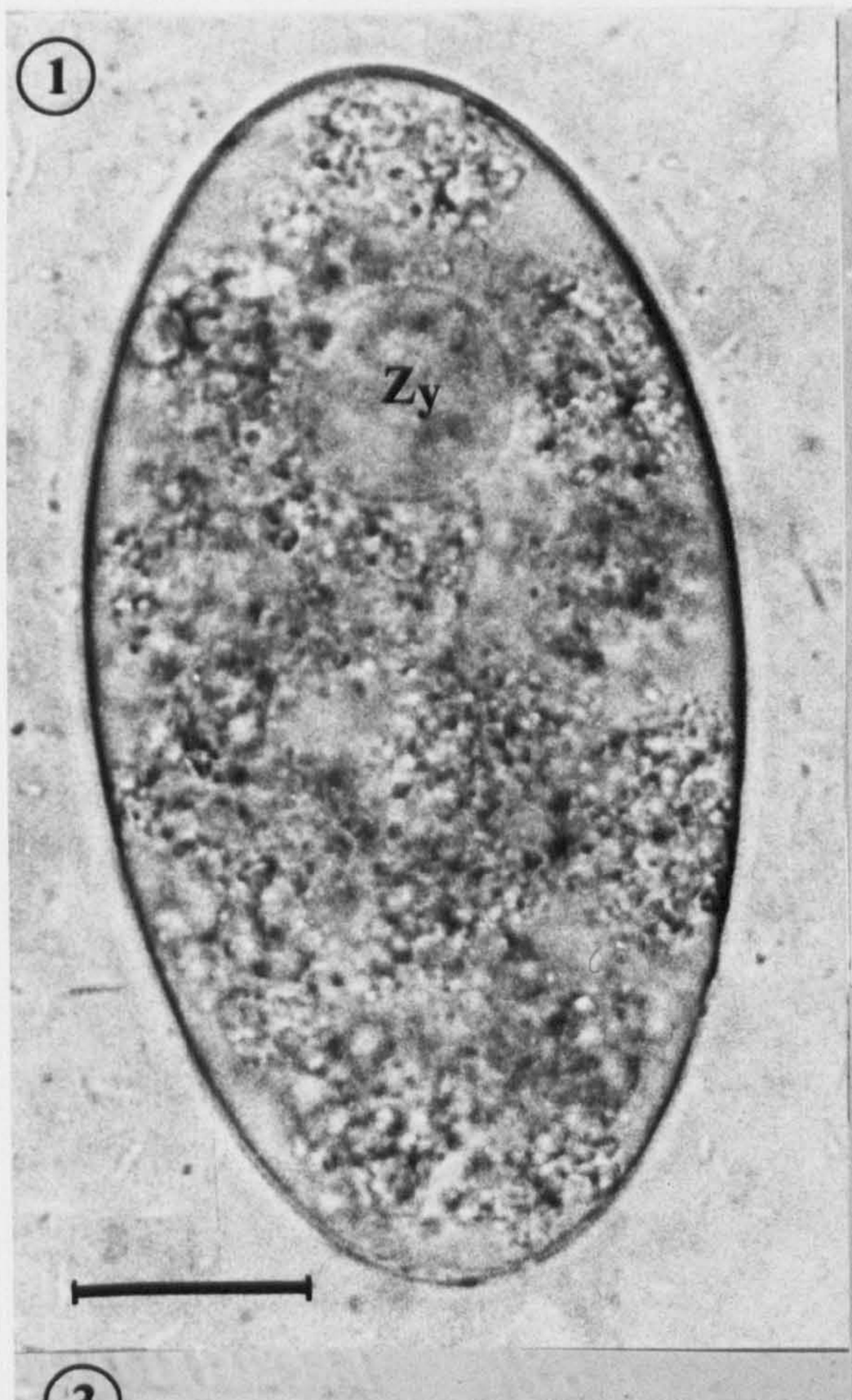
Key

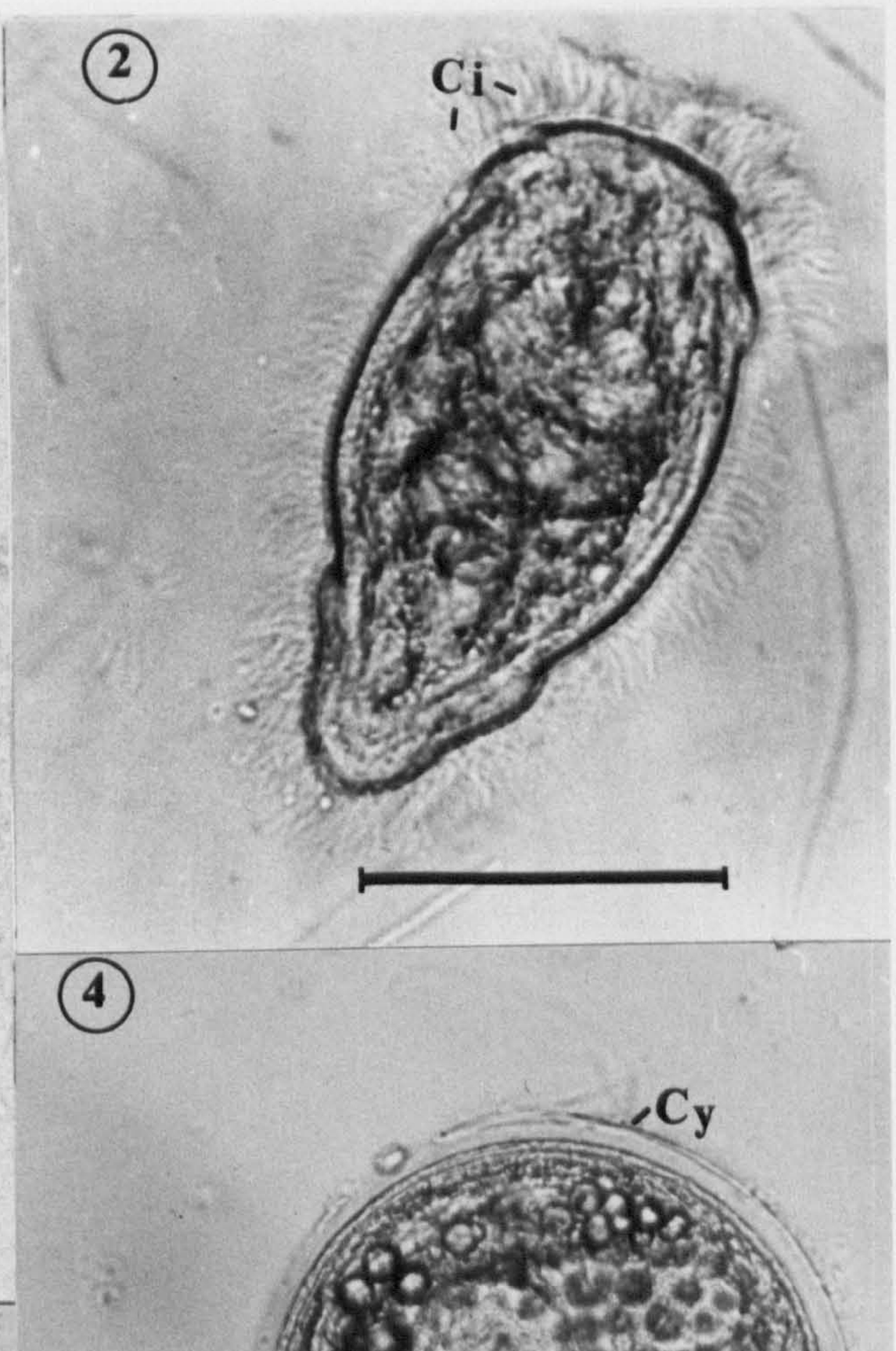
Zy-zygote Ci-cilia T-tail Vs-ventral sucker

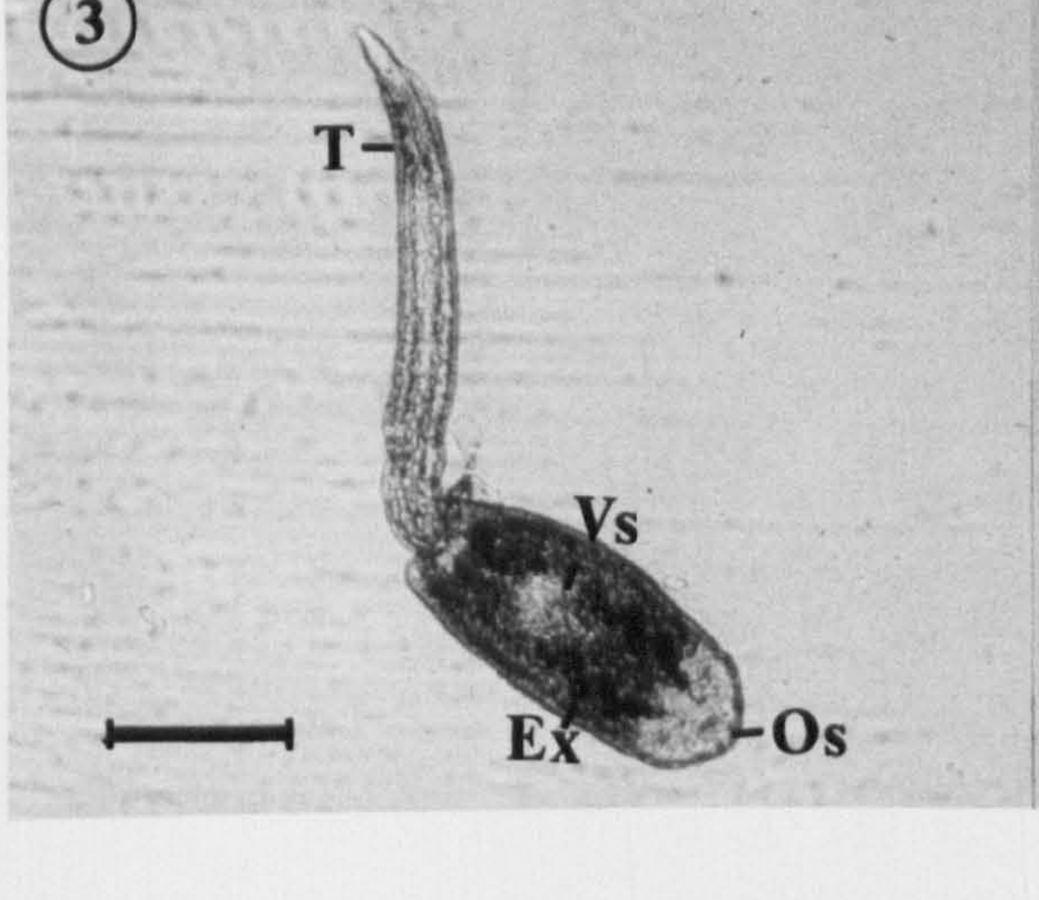
Os-oral sucker

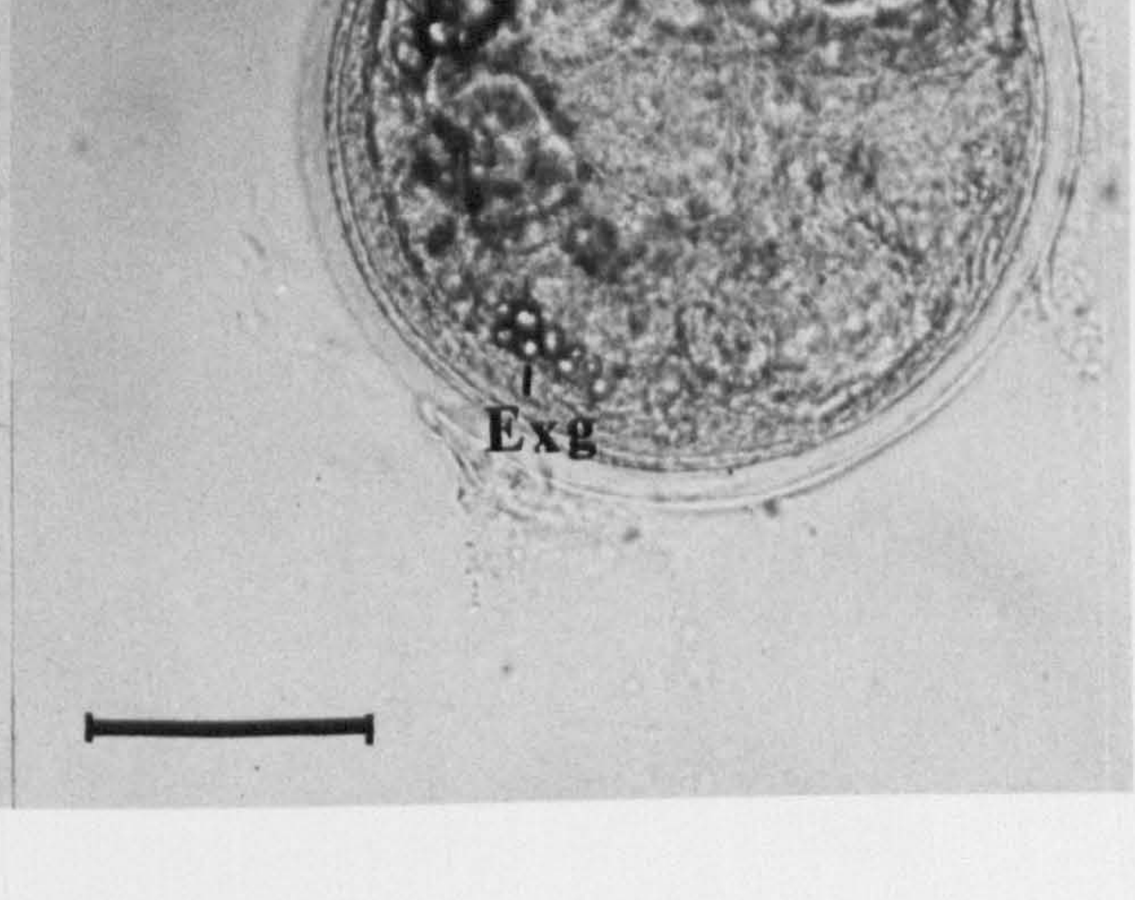
Ex-primary excretory ducts Cy-cyst wall

Exg-primary excretory ducts containing spherical refractile excretory granules


1
Zy


2
Ci


3
T
Vs
Ex
Os


4
Cy
Exg

blood vessels to the ovotestis. Newly released second generation (daughter) rediae develop in the same area. It was not possible to distinguish morphologically between redial generations but large mature rediae appeared orange in colour compared to the colourless appearance of the immature rediae. According to Jeyarasasingam *et al.* (1972) the fully developed second generation rediae contain cercariae which are released from the snail 25 days after miracidial exposure.

Free swimming *E. liei* cercariae (Fig. 1.2 (3)) can infect snails at water temperatures between 19°C and 30°C and their survival period has been demonstrated to decrease with increasing temperature (Evans, 1985). Evans pointed out that transmission efficiencies of both *E. liei* miracidia and cercariae are extremely well adapted for transmission over the range of temperature conditions they are likely to encounter in their natural habitats. The average water temperatures of the Egyptian irrigation canals similar to those habitats occupied by *E. liei* have been found to fluctuate seasonally between 13°C and 30°C (Demian and Kamel, 1972; Moussa and El Hassan, 1972). Ambient water temperatures in sites containing *B. alexandrina* have been reported in the region of 18-22°C (Gohar and El Gindy, 1961) and the optimum range for survival and fecundity is between 20-26°C (Moussa and El Hassan, 1972; El Emam and and Madsen, 1982). Christensen *et al.* (1980) have observed an increase in cercarial production coupled with an increase in the size of *B. glabrata*. These workers also explained that cercarial infectivity to the second intermediate host has been

demonstrated experimentally to include members of the genera *Bulinus*, *Biomphalaria* and *Physa*. Infectivity to the second intermediate host was also found to be independent of the taxonomic identity of the first intermediate host. In *B. glabrata*, cercariae may encyst in the mantle edge but are usually found in the pericardial sac and the nearby kidney spaces (Jeyarasasingam *et al.*, 1972; Kuris and Warren, 1980). It has been demonstrated in the case of the laboratory first intermediate host, *B. glabrata*, that increased snail mortality is evident on cercarial release and re-penetration leading to the snails harbouring all stages of the larval forms (Lim and Heyneman, 1972).

Metacercarial cysts (Fig. 1.2 (4)) which are oval to spherical in shape have been stated to be infectious to the final host 6-7 days after encystment (Jeyarasasingam *et al.*, 1972). The final host infection of *E. liei* is acquired by the consumption of living or dead snails containing infectious metacercariae. The metacercariae excyst in the small intestine and juvenile worms develop to maturity. Eggs appear in the faeces of experimentally infected birds and rodents from 10 days onwards after feeding with infectious metacercariae (Jeyarasasingam *et al.*, 1972). The life span of adult *E. togoensis* in the mouse host has been specified as approximately 6 months by Jourdane and Kulo (1982).

1.3 Echinostomes as biological control agents

The original discovery and description of *E. liei* arose from an investigation of various digeneans to find an agent suitable for the

biological control of *Schistosoma mansoni* and *Schistosoma haematobium* in Africa. Echinostomes have been suggested as possible biological control agents targeted against their intermediate hosts because of their castration effects on these molluscs (Bayer, 1954; Kuris, 1973; Nassi, 1978; Nassi, Pointier and Golvan, 1979). The possibility of the use of echinostomes as biological control agents has also arisen from studies carried out on trematode antagonism in the snail host (Lie, 1967; Lie, Basch, Heyneman, Beck and Audy, 1968; Lim and Heyneman, 1972; Rysavy, Ergens, Groschaft, Moravec, Yousif and El-Hassan, 1973). Later work has shown that several species of *Echinostoma* are able to boost the natural resistance of certain strains of *B. glabrata* to *S. mansoni* (Lie, 1982), while Jourdane, Mounkassa and Imbert-Establet (1990) have demonstrated that during co-infection of *B. glabrata* with *S. mansoni* and *E. liei* the released *S. mansoni* cercariae (before the complete reabsorption of *S. mansoni* sporocysts) show a very marked decrease of their infectivity towards mice.

The interspecific antagonism between larval trematodes coexisting in the tissues of snail hosts has been studied extensively and the laboratory study carried out by Lie, Basch and Hoffman (1967) describes the fundamental features of larval trematode antagonism. These workers exposed the pulmonate snail, *Biomphalaria straminea*, to miracidia of *Paryphostomum segregatum* (27-collar spines) and *Echinostoma barbosai* (37-collar spines), both echinostomes of Brazilian origin. Those snails exposed initially to *E.*

barbosai were found to be easily infected with *P. segregatum*. The original rediae of *E. barbosai* were found to be actively ingested by the developing rediae of *P. segregatum*. The majority of the snails superinfected with *P. segregatum* had their populations of *E. barbosai* eliminated. Snails exposed simultaneously to both of the echinostome species developed as double infections but eventually the *E. barbosai* larvae disappeared while the *P. segregatum* larval forms persisted. When snails infected with *P. segregatum* were later exposed to *E. barbosai*, double infections developed only when the interval between exposure days was 10 days or less. In these infections the *E. barbosai* sporocysts and rediae were confined to the heart ventricle. Lie *et al.* (1967) assumed that the lack of *E. barbosai* forms outside of this location was due to active ingestion by *P. segregatum* rediae. This experimental demonstration of trematode antagonistic qualities by Lie *et al.* (1967) coupled with parasite castration effects (Bayer, 1954; Kuris, 1973; Nassi, 1978; Nassi Pointier and Golvan, 1979) stimulated interest in larval echinostomes as biological control agents. Similarly, the specific interest in *E. liei* is due to its potential as a control agent for schistosomes of medical importance (Kuris, 1980a,b). Heyneman, Lim and Jeyarasasingam (1972) demonstrated that the strong intrinsic competitive nature of both *E. liei* and *P. segregatum* is able to prevent schistosome infections establishing in snails already harbouring these echinostome infections. Heyneman *et al.* (1972) found that *B. glabrata* already harbouring infections of *P. segregatum* were resistant to later infections of *E. liei*. Simultaneous infections of the two

echinostomes showed that *P. segregatum* was the most dominant larval form and no difficulty was found in establishing an infection of *P. segregatum* in *B. glabrata* already harbouring *E. liei*. Interactions between *E. liei* and *Schistosoma mansoni* demonstrated that snails harbouring *S. mansoni* were readily infected with *E. liei* and a rapid loss and degeneration of the schistosome sporocysts followed. The results clearly indicated that *E. liei* miracidia can penetrate and develop in snails at any stages of *S. mansoni* infection. The time of the echinostome development was found to be considerably extended, schistosome cercarial emergence was found to cease after *E. liei* cercariae appeared and doubly infected snails had a short life expectancy. Schistosome miracidia were able to penetrate *E. liei* infected snails but the development of the schistosome larval forms was not successful through to the cercarial shedding stage. Heyneman *et al.* (1972) concluded that *E. liei* can stop schistosome cercarial production either directly or by killing the snail host. They speculated that *E. liei* might be introduced as part of an integrated biological control strategy into schistosome-infected snail populations in Egypt where human schistosome infections were high, with the echinostome populations being maintained from laboratory infections.

The fact that *E. liei* does not already operate as a significant biological control agent under natural conditions in Egypt was put down by Heyneman *et al.* (1972) to a) natural factors which may control the distribution of this parasite preventing it from assuming a major role as a control agent b) a mutual adaption or tolerance

existing which is not detectable under laboratory conditions c) different strains of *B. alexandrina* differing in susceptability to both echinostomes and schistosomes d) and a quantitative difference in the spatial distribution of both *E. liei* and *S. mansoni* possibly existing in Egypt rendering *E. liei* of little importance in controlling human schistosomiasis. A later study by Jourdane and Kulo (1982) considered *E. togoensis* to possess the desirable characteristics for a biological control agent for human schistosomiasis which had been recommended by Combes (1982). These features included the demonstration of a complete and permanent sterilizing effect on *Biomphalaria pfeifferi* by this parasite, snail host mortality in infections of more than five miracidia and a clear dominance of the intramolluscal stages over *S. mansoni*. Other features included the low cost in life cycle maintenance and the high egg productivity of the parasite in the mouse. Kuris (1980a) has also demonstrated the remarkably high level of miracidial searching efficiency of *E. liei* with respect to *B. glabrata*. Such findings lead Combes (1982) to conclude that African echinostomes warranted field trial testing as suitable biological control agents in integrated control programmes directed against intestinal schistosomiasis in Africa alongside the therapeutic treatment of human populations and the use of molluscicides.

Heyneman, Lim and Jeyarasasingam (1972) have explained that field testing of a predatory echinostome must be preceded by analysis of its degree of dominance, suitability, efficiency, possible

danger to man, domestic animals and endangered species and the potential impact it might have on the trematode-snail fauna involved. Obviously great care must be exercised when introducing any biological control agent into the natural environment because of the risks involved in endangering humans directly or indirectly, for example, via infection of animals of nutritional importance. In this context it is relevant to consider the importance of human echinostomiasis. This condition has been reported for the most part from Eastern Asia, most reported infections being of *E. ilocanum*, *E. lindoensis* and *E. revolutum* (Cross and Basaca-Sevilla, 1986). Human infection is acquired from the raw consumption of snails or tadpoles containing infectious echinostome metacercariae (Rim, 1982). Clinical apects of echinostomiasis have been reported by Lie and Virik, (1963) and Seo, Hong, Chai and Lee (1983) and in heavy infections echinostomiasis manifests itself in the form of headaches, gastric pain and diarrhoea (Hilarrio and Wharton, 1917; Yamashita, 1964; Faust, Russel and Jung, 1970). Treatment with mebendazole and praziquantel has been found to be effective (Cross and Basaca-Sevilla, 1986). Interestingly, these workers described how the highly prevalent human infection of *E. lindoensis* in the Lindu Lake region of Central Sulawesi, Indonesia was eliminated from the human population through dietary changes. This involved the introduction of the fish *Talapia mosambica* which led to its consumption rather than the infectious echinostome-carrying clams.

Field trial investigations in Malaysia have involved *E. malayanum* cultured from laboratory infected rats which successfully diminished introduced *Schistosoma spindale* infections (Heyneman and Umathevy, 1967), but in other such field trials microsporidial infections clouded interpretation of the results of the experiments by their selective pathogenicity to the dominant echinostome (Lie, Kwo and Owyang 1970, 1971). Lie, Kwo and Owyang (1971) suggested that the effects of antagonism were noticeable before the microsporidia infection set in. In all instances there was a decrease in the snail population (*Indoplanorbis exustus*), harbouring the digeneans, which was attributed to the lethal effect of double helminth infections. Two further studies carried out in Thailand involved the introduction of *E. malayanum* into ponds carrying *I. exustus* naturally infected with schistosomes. In the first, Lie, Schneider, Sornmani, Lanza and Impand, (1974a) concluded that *S. spindale* control was due to antagonism aided by a marked decrease in the snail population. In the second the failure of the control attempt was ascribed to the submergence of the introduced echinostome eggs by large quantities of mud (Lie, Schneider, Sornmani, Lanza and Impand, 1974b). A further field trial in Guadeloupe demonstrated a decline in number and the sterilisation of naturally occurring *B. glabrata* subjected to the introduction of eggs from the echinostome *Ribeiroia marini guadeloupensis* harvested from the faeces of laboratory rats (Nassi, Pointier and Golvan, 1979).

Combes (1982), discussing echinostome utilization for biological control has listed problems that may reduce the efficiency of such programmes. Some are due to biotic factors such as the interaction of introduced miracidia with non-target organisms and the age structure of the target snail populations. Other non-biotic physico-chemical features of the snails' environment may also adversely affect the efficiency of the introduced echinostome. In addition, there are some theoretical arguments (Anderson, 1978) which suggest that it will be difficult to perturb the existing balance between parasite-induced reduction in snail reproductive potential and tight density-dependent constraints on parasite population growth. Despite these potential problems, Combes called for the continuing investigation of digeneans as biological control agents against schistosomes of human importance. In a report organised through the UNDP/World Bank/WHO special programme for research and training in tropical diseases (1984), on the biological control of snail intermediate hosts, conclusions on parasite antagonistic control suggested that targeting the entire infected mollusc population would be difficult because of the well-known phenomenon of overdispersion. The ultimate objective of antagonistic biological control is that an antagonist fits perfectly into the environment in which it is introduced so that it will effectively and continually maintain control of the target organism. The costs of such operations have not been truly evaluated but recommendations by the "Report of an informal consultation on research on the biological control of snail intermediate hosts" committee (UNDP/World Bank /WHO, 1984) have called for a well-

designed trial in a large transmission site involving the control of *S. mansoni* by the use of digenean antagonism and castration.

1.4 Aims of the present study

The experimental work described in this thesis is all concerned with aspects of the growth, development, reproductive biology and pathogenesis of adults of *E. liei* within laboratory mice. These studies have had a number of separate but interconnected aims. The first relates to the potential use of African 37-spined echinostomes as biological control agents against human schistosome infections in snail intermediate hosts. In this context, *E. liei* has been already extensively studied with respect to the influence of environmental conditions on the larval forms and the impact of intermediate host-related factors on the transmission of the parasite (Christensen, Fransden, Roushdy, 1980; Evans 1985; Kuris 1980a,b). The present study addresses other facets of the echinostome life cycle and epidemiology, specifically those related to the adult worms in a final mammalian host. These studies aim to provide the type of detailed knowledge which is required if we are to be able to predict the impact of enhanced levels of parasitisation within the final hosts that would occur in any integrated control strategy that included the use of *E. liei* in the snail hosts.

Two further objectives of the study stem from the fact that *E. liei* has a remarkably short prepatent period in the mouse host and has life cycle characteristics which enable its maintenance in the laboratory to be achieved with considerable facility. These two

features of the parasite mean that it is an ideal model for the investigation of growth and development of a gut-dwelling digenean within a single generation and also the multi-generation analysis of the reproductive biology of such worms. The latter aspect is made more intriguing because echinostomes like *E. liei* have the potential for self-insemination, so multi-generation studies open up the possiblity of investigation of this component of digenean reproductive success. The *E. liei* /mouse model has been utilized in the present investigation to gain knowledge about all these developmental and reproductive processes.

Finally, in the present confused state of 37-spined echinostome systematics (Kanev, 1985), it is hoped that very detailed laboratory investigations of the adult stages of one of the disputed taxa within this group will aid the final resolution of such taxonomic problems.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 The laboratory maintenance of *Biomphalaria glabrata*

The Egyptian strain of the digenean *Echinostoma liei* was first brought to King's College, London by Dr. N.A. Evans who obtained snails infected with metacercarial cysts from Dr. N.O. Christensen of the Danish Bilharzia Laboratory. The digenean was maintained in an isolate of the tropical freshwater snail *Biomphalaria glabrata* which originated from material obtained in Puerto Rico. This Puerto Rican strain of *B. glabrata* has been cultivated and maintained in the laboratory for at least 10 years. Subsequently *B. glabrata* snails were obtained from the Medical Research Laboratory at Mill Hill, London, the Biology Department of Imperial College, London, and the Zoology Department of the British Natural History Museum to augment the original stocks and to maintain a high level of outbreeding.

2.2 Cultivation of *Biomphalaria glabrata*

B. glabrata are hermaphroditic pulmonate gastropods. Mature snails were kept in plastic aquaria (22 cm x 32.6 cm x 20.5 cm) (width, length and height respectively) containing approximately 8 litres of snail water. Snail water was made up using a hard water synthetic medium (HMSO, 1969) and was aerated for approximately an hour to dechlorinate the final solution (Total hardness 250ppm, pH 7.4). A great variety of methods have been employed by various laboratories for the maintenance of freshwater pulmonate snails. In this study the plastic aquaria were kept in LEEC incubators (LEEC Limited ; CV3 Model and the P33 Extra Large Model) which were maintained at 26°C. In the P33 model the aquaria were continually aerated using a system of air pumps and air stones and the water changed about every 6 to 8

weeks. A population of *Daphnia pulex* were also added to reduce the bacterial growth in the water. In the CV3 Model the snails were kept in plastic trays (23.5 cm x 33.5 cm x 5 cm approx.) for easy access when the snails were required for infection after they were transferred from the large plastic aquaria. Both the aquaria and the plastic trays were covered with a glass lid to prevent the evaporation of water and the escape of snails.

2.3 Water, temperature and light conditioning

During an initial period of snail maintenance on the Strand Campus of King's College, dechlorinated tap water, that is tap water left aerated for 24 hours to allow for the evaporation of chlorine and calcium equilibrium to be obtained, was used for the snail aquaria. On moving to the new Kensington Campus, the water using this method seemed to have a detrimental effect on the snails' condition and so the hard water synthetic medium (HMSO, 1969) was made up using distilled water. To each aquarium 20 to 30 *Daphnia* were added which multiply rapidly at 26°C, because of this, excessive *Daphnia* were removed from the aquaria at intervals when overpopulation occurred.

When the water was changed in the aquaria, at 6-weekly intervals, about two thirds of the water was removed along with the faeces which had collected on the bottom of the aquaria. This was then replaced with fresh artificial hard water. Great care was taken when removing the faeces, as young, recently hatched, snails spent most time on the bottom of the aquaria and feed on the faeces. Young snails in the faeces at water changing were carefully retained. All snails were maintained in incubators at 26°C to secure

optimum development of both uninfected snails and those snails harbouring echinostome infections. Snails maintained in the P33 Extra Large Model had an artificial light cycle installed which provided a light cycle of a 12 hour light period and 12 hour dark period. Snails maintained in the CV3 Model were exposed to natural light.

2.4 Snail feeding

Food provided for the snails consisted of lettuce, commercially known and sold as "round lettuce". Discarding the midrib inner part of the leaf, the leaves were broken into small pieces (about 1 cm across) and placed into the aquaria twice weekly. Guinea pig pellets (Grain Harvester, Ltd.) were used as a food supplement and two pellets added to each aquarium once a week. The quantity of food given to the snails was close to the quantity consumed in 24 hours. Care was taken not to exceed this limit as excessive build-up of lettuce and pellets caused the deoxygenation of the water and the death of both *Daphnia* and snails. The quantity of food added to the aquaria, was adjusted to accommodate the size and number of snails present. Infrequently, unidentified rhabdocoeles, copepods or ostracods established rapidly expanding populations in aquaria. These appeared to make culture conditions less than optimal and many snails crawled out of the water. In these situations the population of the undesirable fauna was eliminated or kept to very low levels by transferring snails to new clean aquaria.

2.5 Snail egg laying and the maintenance of juvenile snails

The laying of egg masses of *B. glabrata* in the laboratory was irregular. Sometimes the snails deposited egg masses daily for

several weeks while at other times they would not lay at all for a number of weeks. In the aquaria just described, the production of juvenile snails was big enough to secure the survival of the stock population and production of enough snails for parasitic infection. The hatching rate was very high but there was also a high mortality rate among newly hatched snails. To maximise survival and growth of newly hatched snails, egg masses were removed from the sides of the aquaria and placed in the plastic trays with other juvenile snails and incubated. One or two mature snails were added to these "nursery snail trays" along with the faeces produced by the adults which acted as a food supplement and increased the survival and growth of the young snails. These juvenile snails were fed on lettuce leaves broken into very small pieces (approximately 5 mm across).

2.6 Infection of *Biomphalaria glabrata*

2.6.1 Miracidial infection

Eggs of *E. liei* were teased from the uteri of adult worms and placed in distilled water. The eggs were held in solid watch glasses filled with distilled water and were completely covered with a large glass slide. The water was changed several times to remove most of the debris and the eggs were then placed in an incubator at 26°C in natural lighting conditions. Over the first eight days the water was continually checked and changed to prevent the formation or build up of any microbial contamination. Nine days after the initial incubation of the eggs they were placed at a distance of 35 cm under a 100 watt lamp between 0800 hours and 1200 hours. After this light stimulus they were held on the bench under ordinary laboratory lighting conditions. Miracidia usually

emerged from 1300 hours onwards but if they did not this process was repeated on days ten, eleven and twelve. Sometimes the hatching was unpredictable but it was usually possible, after exposing the eggs to an artificial light stimulus, to obtain a substantial hatching rate to provide the necessary number of miracidia to infect snails.

Snails were infected in compartmentalised petri dishes (10 cm by 10 cm) divided into 25 compartments, with each compartment measuring 2 cm by 2 cm. Under a binocular microscope a glass pasteur pipette was used to suck up two miracidia which were then placed in each compartment. Snail conditioned water (that is water taken from one of the operational aquaria) was then used to half fill each compartment and into each compartment was then placed a snail measuring between 3 to 5 mm in shell diameter. Larger snails were rarely used but if they measured over 10 mm, 3 to 5 miracidia were added to the compartments. The compartments were then completely filled with snail conditioned water, a transparent plastic lid placed on top, and the whole dish placed in one of the incubators under natural lighting conditions. The next day all the snails that had been exposed to miracidia were placed in the same aquarium. They were fed on both lettuce and guinea pig pellets as described above (Section 2.4) and the aquarium maintained in the same manner as a stock aquarium. Twenty one days postinfection, the snails were individually examined under a dissecting microscope for rediae in the ovotestis. Where the infection was succesful rediae could be seen by transparency through the snail's shell. These snails were then placed back into

the aquarium but examined four days later to check for cercarial emergence.

2.6.2 Cercarial infection

Twenty three days postinfection was the earliest time *E. liei* cercariae were observed being shed from *B. glabrata*. Cercariae, on average, began to emerge from the snails 25 days postinfection. To stimulate shedding infected snails were placed in small plastic (25ml) pots half filled with snail water and placed at a distance of 35 cm under a 100 watt lamp. Within ten minutes cercariae emerged into the surrounding water. Once shedding had begun, individual shedding snails were placed in small (25 ml) plastic pots with 20 ml of snail water and five uninfected snails, to enable the latter to become infected by the cercariae. Exposure of this type was usually continued for 8 hours, followed by a transfer of the snails (shedding and non-shedding) to a larger pot (volume, 500 ml) overnight. This procedure was then repeated for 10 days. After this the previously uninfected snails were removed and maintained to provide a stock of metacercial-infected snails.

2.7 Infection and maintenance of the definitive host

Swiss T.O. (Taylor's Original) outbred male mice were maintained as the definitive host for *E. liei* . Mice were fed water and standard laboratory feed (Grain Harvester Ltd.) *ad libitum*. Metacercarial cysts were dissected from the pericardial sac of *B. glabrata* and were administered orally to six week old mice in 0.1 ml distilled water. Mice were then fed as normal and necropsied when required to obtain adult worms. *E. liei* produces ovigerous worms in the mouse small intestine within eight to nine days postinfection. For

life cycle maintenance, eggs were dissected and embryonated from worms that were aged ten days old or more. Mice carrying echinostome infections aged up to six months could still be used to maintain the life cycle. Mice were killed by cervical dislocation and the intestine rapidly removed and placed in 0.85% saline. The small intestine was then opened with a longitudinal incision and aggregations of adult worms were removed from the mucosa and placed in solid watch glasses containing 0.85% saline. They were then removed washed in distilled water and placed in a solid watch glass containing distilled water. Using fine seekers the eggs were then teased from the uterus of the worms and incubated as described above (see Section 2.6.1).

2.7.1 Collection of faeces

Eggs of *E. liei* were examined in the faeces of mice on a number of occasions during the course of this research programme and a standardized method of counting eggs was adopted. Mice were placed in bottomless cages which contained 0.7% saline in a lower resevoir and left for 24 hours. During this period mouse faeces collected in the saline, which kept the faecal pellets moist and prevented them from dessicating. At the end of the 24 hour period, the faecal pellets were collected and blotted with filter paper. The faeces were then pooled and weighed as one sample.

2.7.2 Examination of faecal smears

After the faeces had been weighed and mixed together as an even paste a random sub-sample was taken corresponding approximately in size to two faecal pellets and weighed. This sub-sample was then evenly suspended in water to make a final

volume of 2 mls and kept suspended by constant stirring. Using a micropipette, 100 microlitre aliquots were taken from the stirred suspension and examined microscopically for *E. liei* eggs. Counting eggs in an appropriate number of 100 microlitre aliquots from the original volume enabled the egg output by the worms in 24 hours to be estimated.

2.8 Preparations of specimens for light microscopy

A number of light microscopical staining techniques were used during the course of this work to study the morphology and development of *E. liei*.

2.8.1 Whole mount preparations: Borax carmine

Borax carmine was used as a general stain for whole mount preparations of *E. liei* to provide a general insight into the worm's morphology. The borax carmine was prepared as described in Appendix 1, allowed to settle for a day and then filtered. Specimens for staining were fixed in 70% alcohol for two days and then placed in borax carmine. This staining period varied from specimen to specimen but usually varied from 15 to 30 minutes. After staining, the specimens were differentiated in 50% acid alcohol and left until the appropriate point of differentiation had been reached. This point was taken as the faintest pinkish colour possible. Subsequent treatment involved dehydrating the specimens through a graded series of alcohols starting at 70% and then clearing in xylene. They were then left in a mixture of half xylene and half Ralmount (BDH) overnight to facilitate the impregnation of the mounting medium. The next day the specimens were mounted in pure Ralmount.

2.8.2 Staining of the vitelline glands: Fast red salt B

The stable diazotate of 5 nitro-anisidine, available commercially as Fast Red Salt B (Sigma) was used to examine the development of the vitellaria in whole mount preparations of the developmental stages of *E. liei*, using the azo coupling method. Worms were fixed in 70% alcohol for 1 to 3 days or longer and then taken through a decreasing series of alcohol concentrations to distilled water and then stained in freshly prepared 1% Fast Red Salt B (See Appendix 1). Staining was rapid and so all specimens were left in the stain for 5 minutes to standardise the proce dure. After this time the specimens were taken up an alcohol series, cleared in xylene and then left in a mixture of half xylene half Ralmount overnight. The next day the worms were mounted in pure Ralmount.

2.8.3 Staining of sections

Specimens of *E. liei* for sectioning were stained in Mayers haematoxylin and eosin. Specimens were fixed in formyl acetic acid alcohol (FAA) (See Appendix 1) for two days or more and then taken through a graded series of alcohols to xylene impregnated with wax, followed by wax embedding and sections measuring 6 µm in thickness were cut using a rotary microtome. Staining was carried out using standard haematoxylin and eosin staining techniques (See Appendix 1).

2.8.4 Chromosome squashes

To examine the chromosomes of *E. liei*, chromosome squashes of certain tissues were made by staining fresh specimens in aceto-orcein solution (See Appendix 1) for 15 to 30 minutes. The specimens were then removed to a fresh batch of stain and

squashed between two slides. For material that was not needed immediately, specimens were stored in Carnoy's solution (See Appendix 1) Before carrying out aceto-orcein squashing on stored specimens, they were placed in acid alcohol for 2 to 3 minutes, returned to Carnoy's for 5 minutes and then stained in aceto-orcein.

2.9 Preparation of specimens for electron microscopy

Scanning and transmission electron microscopical techniques were used during the course of this research to study certain ultrastructural aspects of *E. liei*. Worms were dissected from infected mice and were treated as described below for scanning and transmission work.

2.9.1 Scanning electron microscopy

Two methods were used for the preparation of specimens for scanning electron microscopy. Initially specimens were critical-point dried but later specimens were freeze dried. Specimens that were critical-point dried were fixed in 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.2) at room temperature for four hours and then dehydrated through a graded series of acetone/water mixtures up to absolute acetone and critical-point dried (EM Scope CPD750). The specimens were then coated with gold using a Polaron E5100 Sputter Coater and placed on stubs for examination with a JEOL JSM 25S scanning electron microscope. Freeze dried specimens were also fixed in 2.5% glutaldehyde in 0.1M sodium cacodylate buffer after a brief wash in distilled water. Immediately before freeze drying, specimens were washed in distilled water and then freeze dried in the EMSCOPE Model FD500, gold coated and then examined as described above.

2.9.2 Transmission electron microscopy

Worms for transmission electron microscopy were also fixed in 2.5% glutaldehyde in 0.1M sodium cacodyate buffer (pH 7.2) at room temperature for two hours and then placed in a fresh solution and left overnight. They were then washed in 0.1M sodium cacodylate buffer for two hours (this buffer was ideal for long term storage of the specimens if they were not required immediately). The specimens were post fixed in 1% osmium tetroxide in 0.1M sodium cacodylate buffer for two hours and then dehydrated through a graded series of alcohols and cleared in propylene oxide for thirty minutes. The specimens were then infiltrated with Spurr resin (See Appendix 1) over three days. Initially this involved spinning equal amounts of Spurr resin with propylene oxide at 1 rpm for one hour and then adding the total volume again of Spurr resin and spinning (1 rpm) for 24 hours to facilitate impregnation of the speci mens with the resin constituents. After this time specimens were placed in pure resin and spun (1 rpm) for two days. These specimens were then embedded in moulds in fresh Spurr resin and left in an oven at 60°C for two days. Following polymerisation of the resin, thin sections (40-60 nm) were cut with a diamond knife with a Sorvall MT2B Porter Blum ultramicrotome, placed on copper grids and stained with uranyl acetate at 60°C for ten minutes, washed with distilled water and then stained with lead citrate at room temperature for ten minutes. Sections were examined and photographed in a JEOL Model 100.

CHAPTER 3

THE SOMATIC GROWTH AND DEVELOPMENT OF *ECHINOSTOMA LIEI* IN THE MOUSE LABORATORY HOST

3.1. Introduction

The infectivity, somatic growth and development of a number of digenean species has been studied in the past. Among the echinostomes, studies have concerned *Echinostoma revolutum* (Beaver 1937; Fried, 1984; Hosier and Fried, 1986; Franco, Huffman and Fried, 1986), *Echinoparyphium recurvatum* (Senger, 1954) and *Echinostoma hortense* (Saito, 1984). Beaver (1937) studied *E. revolutum* in naturally and experimentally infected avian and mammalian definitive hosts while Senger (1954) studied the growth, development and survival of both *E. revolutum* and *E. recurvatum* in chicks and rats. Senger (1954) used three quantitative criteria, body length, fresh weight and dry weight to determine the rate of growth of these echinostomes in their definitive hosts. Both Beaver (1937) and Senger (1954) selected species that matured quickly and were characterised by a rapid early development in the definitive host. Fried (1984) carried out a similar study on the infectivity, growth and development of *E. revolutum* in the domestic chick while Saito (1984) looked at the development of *E. hortense* in Wistar rats and used measurements of the genital organs to construct growth curves. Moravec, Barus, Rysavy and Yousif (1974) made observations on the development of the Egyptian strains of *E. recurvatum* and *E. revolutum* while Franco, Huffman and Fried (1986) and Hosier and Fried (1986) studied the infectivity and growth of *E. revolutum* in the golden hamster, *Mesocricetus auratus* and Swiss Webster and ICR mice respectively. With respect to *E. liei*, Fried and Emili (1987) compared the growth and infectivity of *E. revolutum* and *E. liei* in chicks, concluding that the rate of growth and development was greater in the former than in the latter while a further study by

Fried, Donovick and Emili (1988) looked at the growth and development of *E. liei* in chicks over 28 days postinfection. Non-echinostome studies have included that conducted by Nollen (1971) who observed the growth and infectivity of *Philophthalmus megalurus* in chicks and concentrated on increases in length as an expression of growth. Similarly, Dawes (1962) infected mice with metacercarial cysts of the sheep liver fluke *Fasciola hepatica* and examined worms over the period from the first day of infection to maturity and made possible for the first time a study of growth and maturation in this digenean.

The benefits of a histochemical approach to the study of development in digeneans were originally pointed out by Bell and Smyth (1958). They used cytological and histochemical criteria to recognise developmental phases in both trematodes and pseudophyllidean cestodes. Senger's (1954) earlier work had involved stained whole mounts of *E. revolutum* and *E. recurvatum* for morphological studies and to determine the extent of development that had taken place. His report also included comments on the appearance of eggs in the uterus. Fried and Morrone (1970) made reference to the identification and distribution of lipids in *E. revolutum* recovered from the chick intestine, from chorioallantois culture and from worms that had been starved in a non-nutrient medium. They used numerous staining techniques but did not utilize them to investigate developmental changes. A later study by Fried and Stromberg (1971) made reference to the presence of phenolase, phenols and basic proteins in *E. revolutum*. A further study by Fried (1985), involved the examination and the localization of alkaline

phosphatase activity in the digenean *Leucochloridiomorpha constantiae* over its early development on the chick chorioallantois, while Breckenridge and Nathanael (1988) used numerous histochemical techniques to detect protein, phenols and phenolase in the vitelline glands of the commensal temnocephalid *Paracaridinicola platei*.

Numerous scanning electron microscopy (SEM) studies have been carried out on a number of digeneans to observe their surface topography. Bennett (1975) examined *Fasciola hepatica* during its growth and development in the mouse. Hoole and Mitchell (1981), made ultrastructural observations on the sensory papillae of juvenile and adult *Gorgoderina vitelliloba*, while Thulin (1980) examined the sanguinicolid, *Aporocotyle simplex*. Schistosome surface SEM investigations have included studies conducted on adult worms of *Schistosoma leiperi* (Southgate, Ross and Knowles, 1981) and adult worms of *Schistosoma margrebowiei* (Ogbe, 1987). With respect to the echinostomatid digeneans work is limited but includes at least five useful studies. Smales and Blankespoor (1984) undertook a detailed study of the surface topography of *E. revolutum* and *Isthmiophora melis* adults while Fried and Fujino (1984) studied the early development of *E. revolutum* in the chick embryo and the domestic chick. Koie (1987) observed the surfaces of adults and juveniles of the echinostome *Mesorchis denticulatus*. while Tesana, Kanla, Maleewong and Kaewkes (1987) similarly studied adults of *Echinostoma malayanum*. A recent unrefereed abstract by Fried, Irwin and Lowry (1989) commented on SEM observations on the surfaces of adults of both *E. revolutum* and *E. liei*.

In this study, the infectivity of *E. liei* was assessed by recording the establishment success of this parasite in its definitive host. Quantitative criteria used to assess growth and development were body length, maximal body width, absolute body area, vitelline gland area and oral sucker and ventral sucker diameters. Qualitative criteria used to assess growth included the identification of the presence of egg shell precursors and development of the organ structures as revealed by light microscopy. Alongside this, the tegumental surface features of *E. liei* were examined with SEM to provide a basis for comparison with those echinostomes already studied by this methodology.

3.2 Materials and Methods

3.2.1 Recovery of worms for growth analysis

Thirty, six week old male mice were each infected with 25 metacercarial cysts of *E. liei* and killed on successive days postinfection from day 4 to day 10 and then on days 15, 20 and 100. Three replicates were killed at each of the specified days postinfection. Recovery of worms infected with 25 metacercarial cysts on days 1, 2 and 3 postinfection was very difficult because of the worms small size and mixing with intestinal contents. For this reason infection levels were increased to 50 metacercarial cyst and hence nine six week old mice were infected with 50 metacercarial cysts of *E. liei* and killed on the first three days postinfection.

Mice were killed by cervical dislocation and the small intestine removed for worms recovered from days 4 to 100 postinfection. At these successive days postinfection the intestine was searched for

parasites, using the high power of a binocular microscope particularly in the case of the worms up to 7 days postinfection. The peritoneal cavity was also searched for worms along with the large intestine, liver, spleen, kidneys and bladder. For days 1 to 3 postinfection at necropsy, the intestines were flooded with Bouin's fixative (See Appendix 2) and searched for worms using the high power of a binocular microscope. This procedure had been found to facilitate visualisation of these juvenile worms.

Worms recovered on days 4 to 100 postinfection that were to be used for light microscopy were immediately washed in 0.85% mammalian saline and then fixed briefly in Berlands solution (see Appendix 1) for no more than ten seconds, and then fixed in 70% alcohol in which they were stored until required. Worms that were fixed in 70% alcohol after fixation in Berlands solution and were complete and relatively flat had their body lengths and maximal widths measured using a calibrated eyepiece micrometer. Ventral sucker and oral sucker measurements were also made on fixed worms on days 2, 3, 6, 7, 8, 10, 15, 20 and 100 postinfection.

A random sub-sample of fixed worms from 4 to 20 days postinfection used for body length and maximal width measurements, were then stained completely in Erlich's haematoxylin (Sigma) and left in the stain overnight so that the worms appeared completely black in colouration.

To study vitelline gland development a further sub-sample of worms aged 4-20 days postinfection that had been fixed in 70% alcohol were stained with the stable diazotate, 5-nitro-anisidine,

known and commercially sold as Fast Red Salt B (See Section 2.8.2). Worms were taken down an alcohol graded series to distilled water and stained in 1% Fast Red Salt B for 5 minutes.

Worms stained with Erlich's haematoxylin and Fast Red Salt B were then used for the calculation of whole body area and vitelline gland area measurements respectively. These area measurements were carried out with the use of the Watford BBC Video Digitiser (Watford Electronics) and a purpose-written built programme (Name "AREA". Details of this programme listing may be obtained from Andrew Langridge, Biosphere Sciences Division, Kings College, London, the author of this programme). Using a composite video (JVC GX-N7E) and the Watford BBC (with a resolution of 640 by 256 pixels) the image of the worms was converted into a graphic screen. This enabled the area occupied by the worms to be calculated in arbitary units. The calibration of the digitiser involved recording the area occupied by an object of known dimensions hence providing the necessary information to convert the calculated worm areas to actual units.

Whole mount preparations for light microscopical developmental observations were made by staining worms with Borax carmine following the procedure outlined in Appendix 1 after they had been fixed in 70% alcohol. Worms for electron microscopy studies were immediately teased from the mucosa and fixed in 2.5% glutaldehyde in 0.1M Sodium cacodylate (pH 7.2) without any preliminary washing in mammalian saline and prepared for SEM as detailed in Section 2.9.1.

3.3 Results

3.3.1 Infectivity and growth of *E. liei*

The infectivity and growth data are summarized in Tables 3.1, 3.2, 3.3 and 3.4. Table 3.1 contains the mean worm establishment for each replicate group from an infection dose of 25 or 50 metacercarial cysts while Table 3.2 and 3.3 contain the data for body lengths and maximal body widths and the measurements for the oral and ventral suckers respectively. Table 3.4 contains the worm area and vitelline gland area data provided by the Watford BBC digitiser.

Infectivity

All mice became infected and harboured worms at each of the infection days studied. These worms were confined exclusively within the small intestine. The number of worms recovered from the group of mice administered 25 metacercarial cysts ranged from 5 to 25. Of this group, from the 750 cysts administered to the mice 410 (54.7%) were recovered as worms from days 4 to 100 postinfection. In the smaller group of mice administered 50 metacercarial cysts the number of worms recovered ranged from 7 to 21. From the 450 metacercarial cysts administered in total to these mice 112 (24.9%) were recovered from days 1 to 3. Fig. 3.1 shows the relationship between apparent establishment percentages and days postinfection. The values rise up to levels of between 44% and 65% by day 5 and remain at this level until day 100. This rise phase from day 1 to 4 is presumably artefactual as it is obvious that actual establishment levels cannot rise during the course of an infection. This issue will be considered in more detail in the Discussion section of this Chapter.

TABLE 3.1 The establishment success of E.liei at successive days postinfection

Days	Metacercarial cyst dose	Mean number of worms recovered	Apparent mean percentage establishment	95% Conf.limits of apparent mean % establishment (+/-)
1	50	9.3	18.7	4.6
2	50	12.3	24.7	8.3
3	50	15.7	31.3	10.1
4	25	9.3	37.3	4.6
5	25	13.7	54.7	6.9
6	25	16.0	64.0	12.1
7	25	12.0	48.0	6.6
8	25	11.0	44.0	5.8
9	25	14.7	58.7	18.4
10	25	16.0	64.0	12.9
15	25	16.3	65.3	12.2
20	25	11.3	45.3	15.4
100	25	16.3	65.3	10.8

TABLE 3.2 The mean length and width of E.liei at successive days postinfection

Days postinfection	Length of worm (mm)	* 95% Confidence Limits (mm)	Maximal width of worm (mm)	^95% Confidence Limits (mm)	**No. of worms
1	0.280	0.057	0.090	0.001	12
2	0.610	0.009	0.200	0.004	21
3	0.910	0.023	0.240	0.008	25
4	1.280	0.094	0.250	0.029	18
5	2.200	0.119	0.400	0.022	20
6	3.040	0.127	0.650	0.018	19
7	3.830	0.165	0.820	0.030	16
8	4.460	0.181	1.020	0.036	30
9	5.420	0.506	1.270	0.049	22
10	5.840	0.120	1.370	0.016	20
15	6.050	0.301	1.500	0.061	20
20	6.330	0.283	1.690	0.078	25
100	9.340	0.210	2.080	0.030	29

* 95% Confidence limits of the mean worm length
** Number of worms for both width and length measurements
^95% Confidence limits of the mean worm width

TABLE 3.3 The mean diameter of the oral and ventral suckers of E.liei at successive days postinfectior

Days postinfection	Oral sucker (mm)	95% Confidence limits (mm)	Ventral sucker (mm)	95% Confidence limits (mm)	Number
2	0.044	0.000	0.088	0.000	8
3	0.066	0.000	0.118	0.008	10
6	0.105	0.010	0.212	0.016	8
7	0.105	0.009	0.230	0.009	8
8	0.125	0.014	0.355	0.014	8
10	0.123	0.008	0.367	0.022	11
15	0.128	0.018	0.429	0.016	9
20	0.142	0.008	0.450	0.007	8
100	0.150	0.002	0.520	0.020	10

TABLE 3.4 The body area and vitelline gland area of E.liei at succeessive days postinfection

Days postinfection	Body area (sq.mm)	*95% Confidence limits	*Number	Vitelline gland area (sq.mm)	^95% Confidence limits	^Number
4	0.23	0.03	10	0.00	0.00	
5	0.83	0.13	10	0.00	0.00	
6	1.51	0.05	6	0.00	0.00	
7	1.98	0.19	8	0.00	0.00	
8	2.97	0.30	10	0.37	0.05	8
9	3.97	0.56	10	0.45	0.04	11
10	4.64	0.21	10	1.12	0.17	11
15	4.75	0.22	10	1.83	0.35	9
20	4.90	0.54	10	1.83	0.22	10

* 95% Confidence limits and worm number for body area
^95% Confidence limits and worm number for vitelline gland area

Length and width growth

The relationship between mean length and parasite age is expressed graphically in Fig. 3.2 with 95% confidence limits of the means and similarly for their widths over 20 days post-metacercarial cyst infection (see Table 3.2). The growth in length and width of the worms over 100 days is shown in Fig. 3.3 as a semi-log plot. Comparisons of these curves indicate that growth with respect to these two criteria adopts the shape of a sigmoid curve. During the development of *E. liei* the mean worm length increased from 0.28 mm on day 1 to 6.33 mm on day 20 and the width from 0.09 mm to 1.69 mm on days 1 and 20 respectively indicating rapid growth in both these attributes over this 20 day period when compared with growth from days 20 to 100. Fig. 3.4 shows that over the full 100 day development period analysed, growth in terms of length and width was allometric, the two attributes changing in direct proportion to one another ($R=0.990$; $P<0.001$; $N=13$). Over the first 20 days however growth was clearly non-allometric with the witdh increasing more rapidly than length.

Sucker growth

Oral and ventral sucker measurements are represented graphically in Fig. 3.5 from days 2 to 100. The diameter of the oral sucker increases from 0.044 mm on day 2 to 0.150 mm on day 100 while the ventral sucker increases from 0.088 mm on day 2 to 0.520 on day 100. In both cases a sigmoid shaped curve is apparent. Fig. 3.6 shows the relationship between oral and ventral sucker diameters in the period examined between 2 and 100 days. It reveals a pattern of non-allometric growth with respect to these two

Fig. 3.1

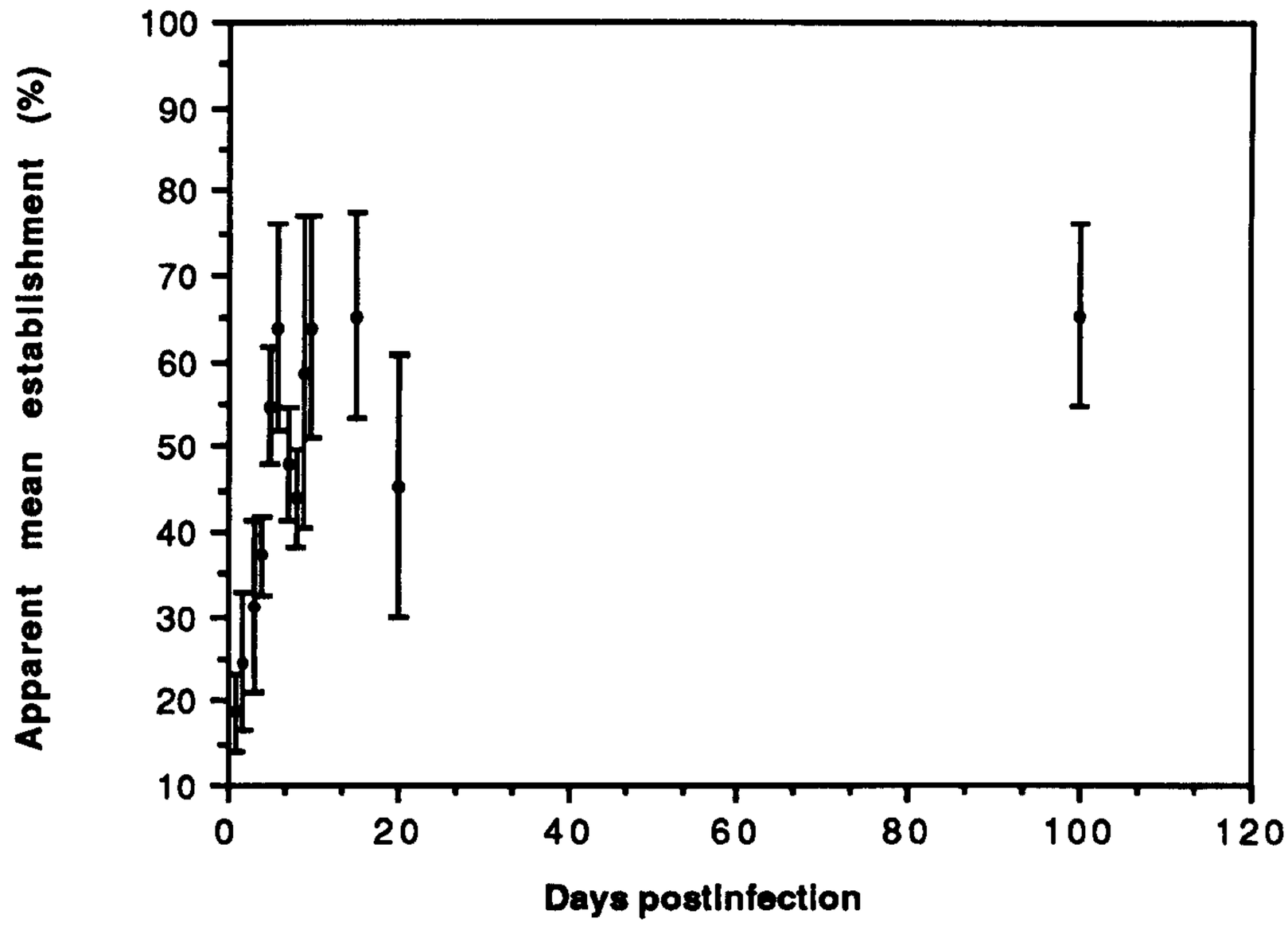


Fig. 3.1 Apparent mean establishment

(data points include 95% confidence limits)

Fig 3.2 Mean length and width at successive days postinfection (data points include 95% confidence limits)

Fig. 3.3 Semi-logarithmic plot of mean length and width

Fig. 3.2

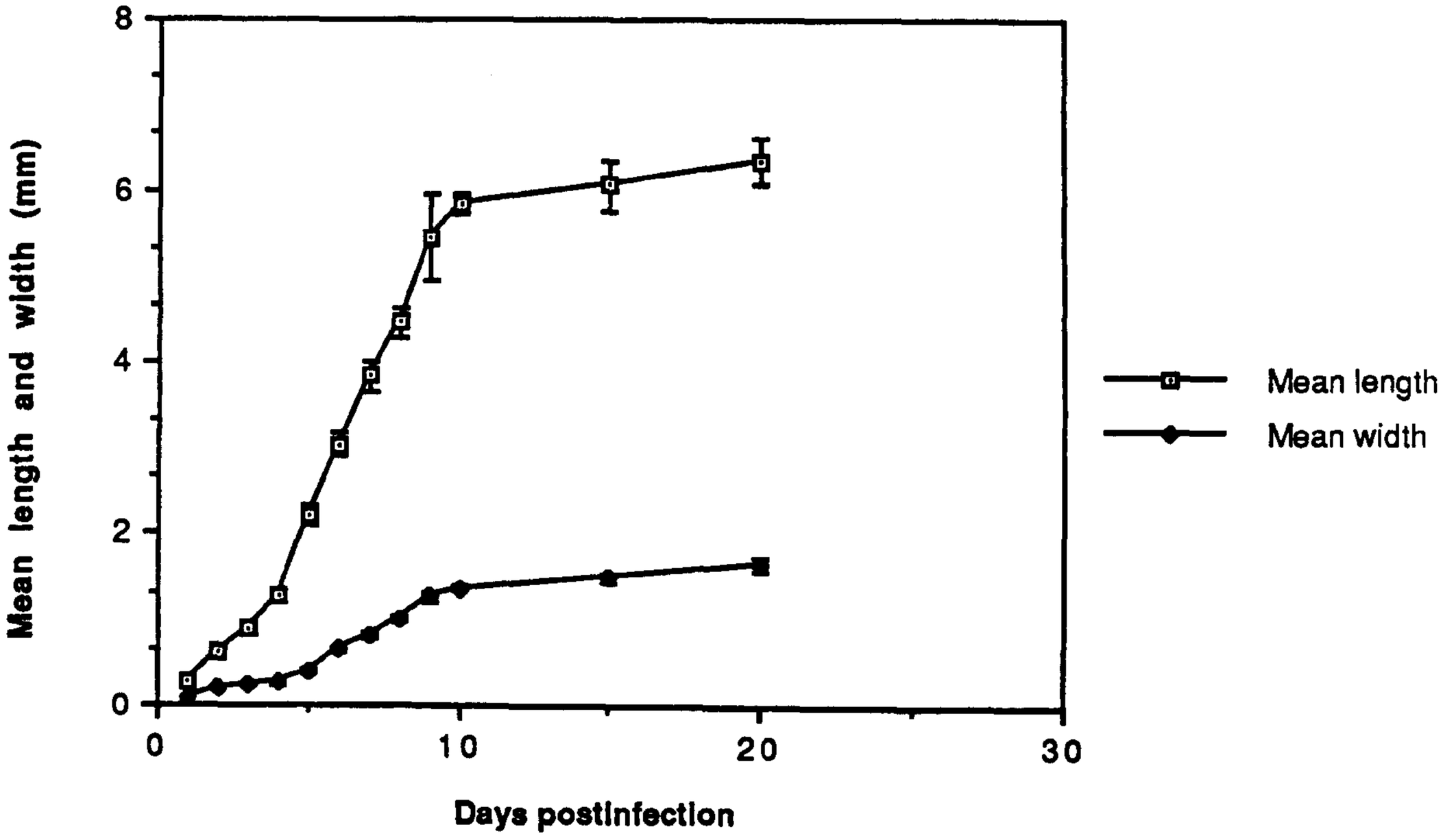

Mean length and width (mm)
8
6
4
2
0
0
10
20
30
Days postinfection
Mean length
Mean width

Fig. 3.3

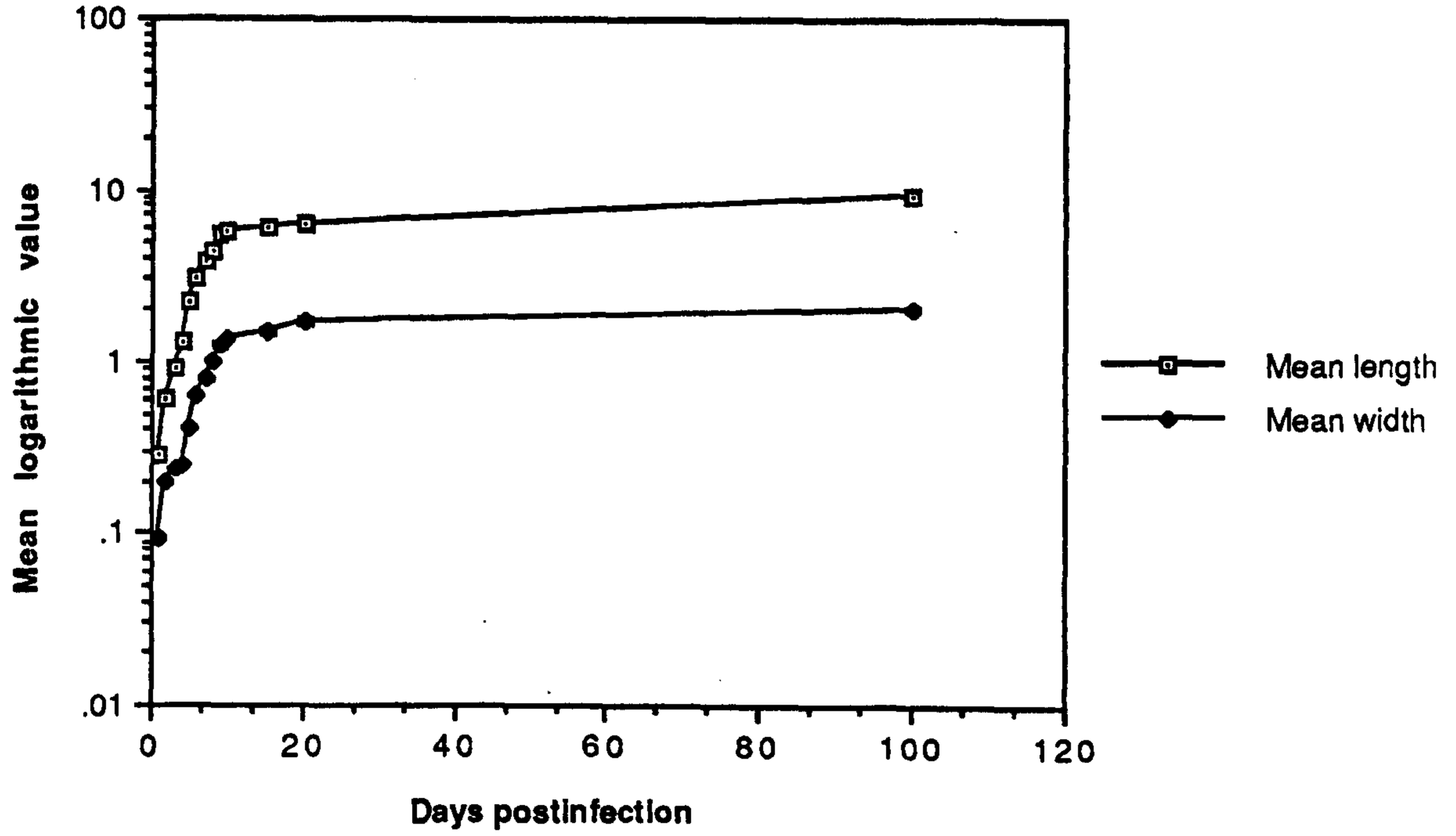

Mean logarithmic value
100
10
1
.1
.01
0
20
40
60
80
100
120
Days postinfection
Mean length
Mean width

Fig. 3.4 The relationship between length and width

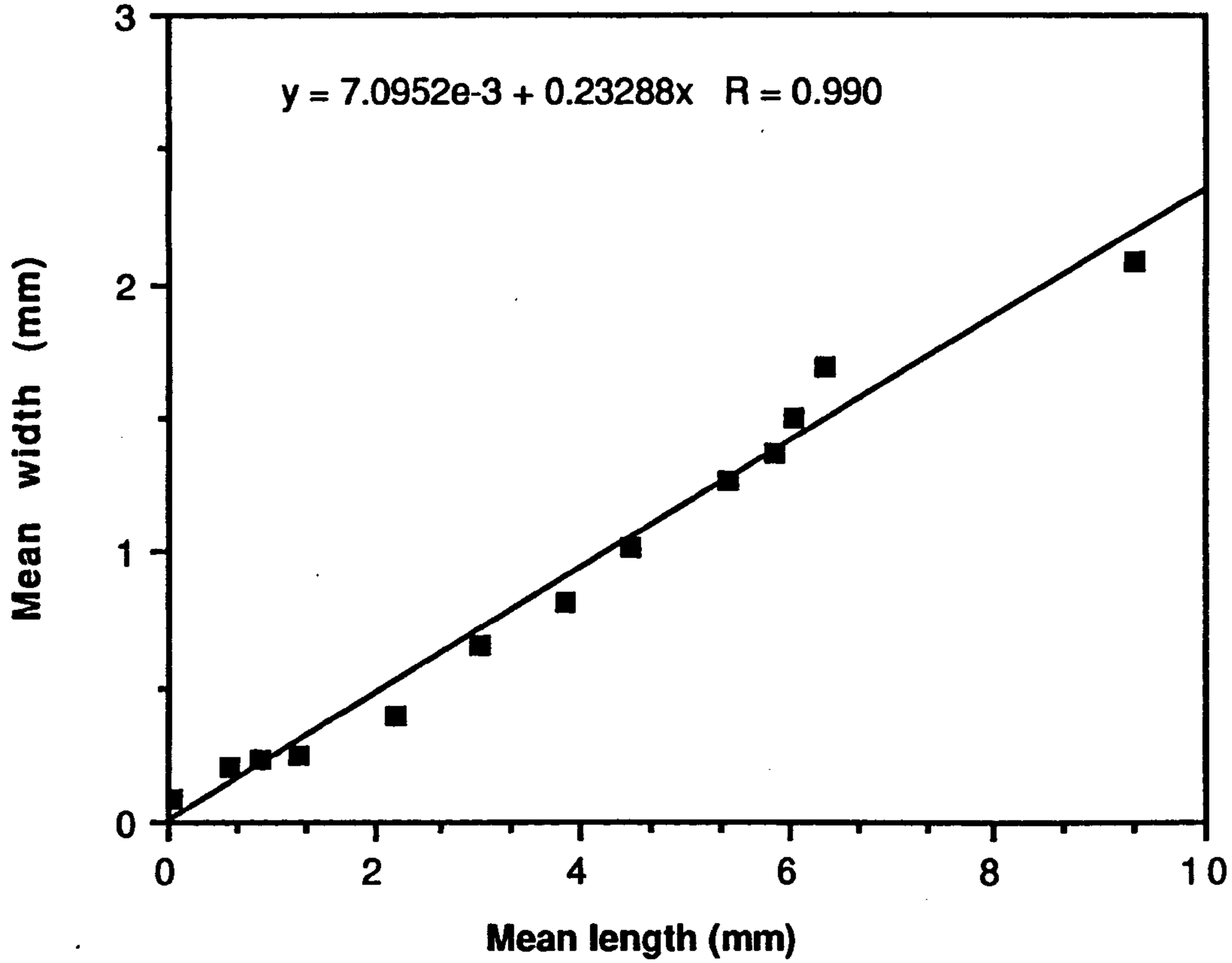


Fig. 3.5 The mean diameters of the oral and ventral suckers (data points include 95% confidence limits)

Fig. 3.6 The relationship between the oral and ventral suckers

Fig. 3.5

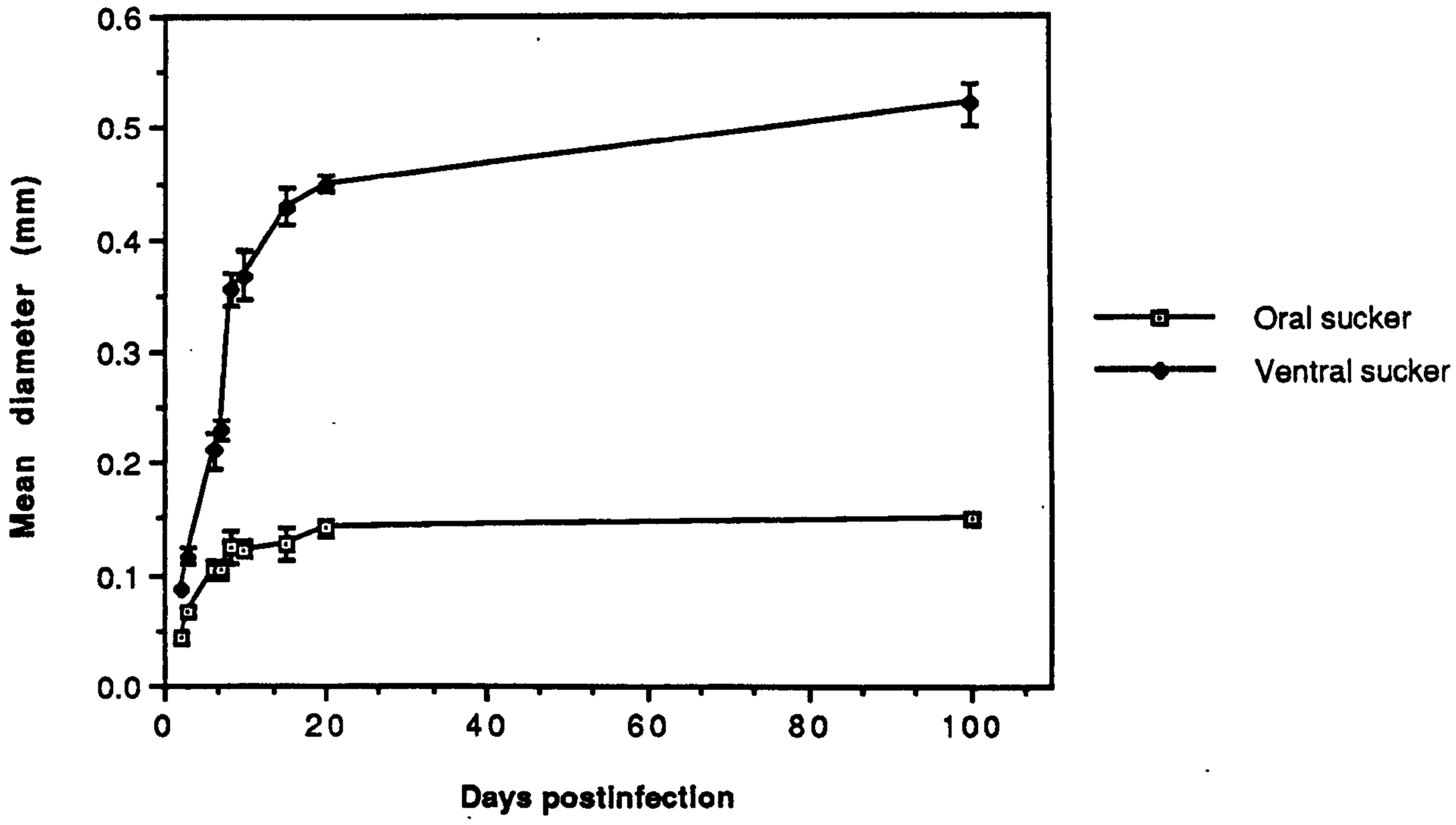
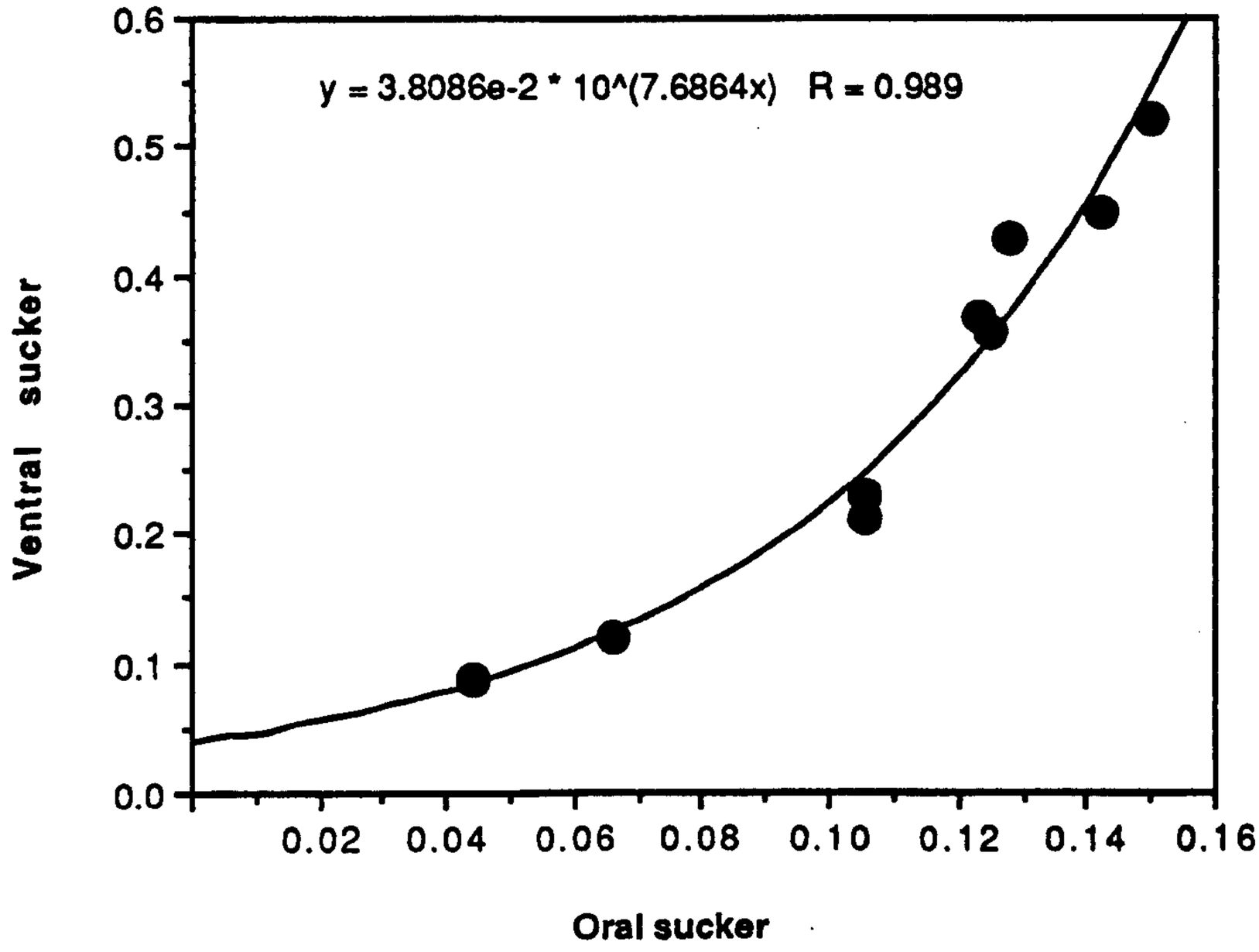

0.6
0.5
0.4
0.3
0.2
0.1
0.0
Mean diameter (mm)
0
20
40
60
80
100
Days postinfection
Oral sucker
Ventral sucker

Fig. 3.6


y = 3.8086e-2 * 10^(7.6864x) R = 0.989
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Ventral sucker
0.02
0.04
0.06
0.08
0.10
0.12
0.14
0.16
Oral sucker

measurements with the ventral sucker increasing more rapidly in size than the oral sucker (R=0.989; P<0.001; N=9).

Area growth

The growth in area (see Table 3.4) of *E. liei* from days 4 to 20 postinfection appears to mirror that of width and length increases and exhibits the same essentially sigmoid curve and is shown graphically in Fig. 3.7 with attached 95% confidence limits of the means. From Table 3.4 it can be seen that there is a near 4 times increase in the proportional body area of *E. liei* between days 4 and 5 (0.23 mm^2 to 0.82mm^2), a near double in size between days 5 and 6 (0.82 mm^2 to 1.51 mm^2) and from days 10 to 20 the proportional size changes are negligible. A graphical representation of the instantaneous area growth rate against days postinfection is shown in Fig. 3.8 (R=0.922; P<0.001; N=9). Instantaneous area growth rates were estimated from the relationship ($Log_n A_{T2}$-$Log_n A_{T1}$)/T2-T1 where A_{T2} is the area at time T2 and A_{T1} is the area at time T1. This value of the instantaneous growth rate was then assigned to the mean point of this time interval, that is, (T1+T2)/2. Fig. 3.8 clearly depicts an exponential decline in the instantaneous rate of growth throughout the period sampled (days 4-20 postinfection).

Vitelline gland growth

The diazotate Fast Red Salt B procedure carried out on worms aged 4 to 20 days postinfection identified vitelline precursors from 8 days postinfection onwards only, therefore area studies identified the vitelline fields on days 8, 9, 10, 15 and 20 postinfection (see Table 3.4). These vitelline areas are plotted graphically in Fig. 3.9

Fig. 3.7 Mean body area at successive days postinfection (data points include 95% confidence limits)

Fig. 3.8 Area instantaneous growth rate

Fig. 3.7

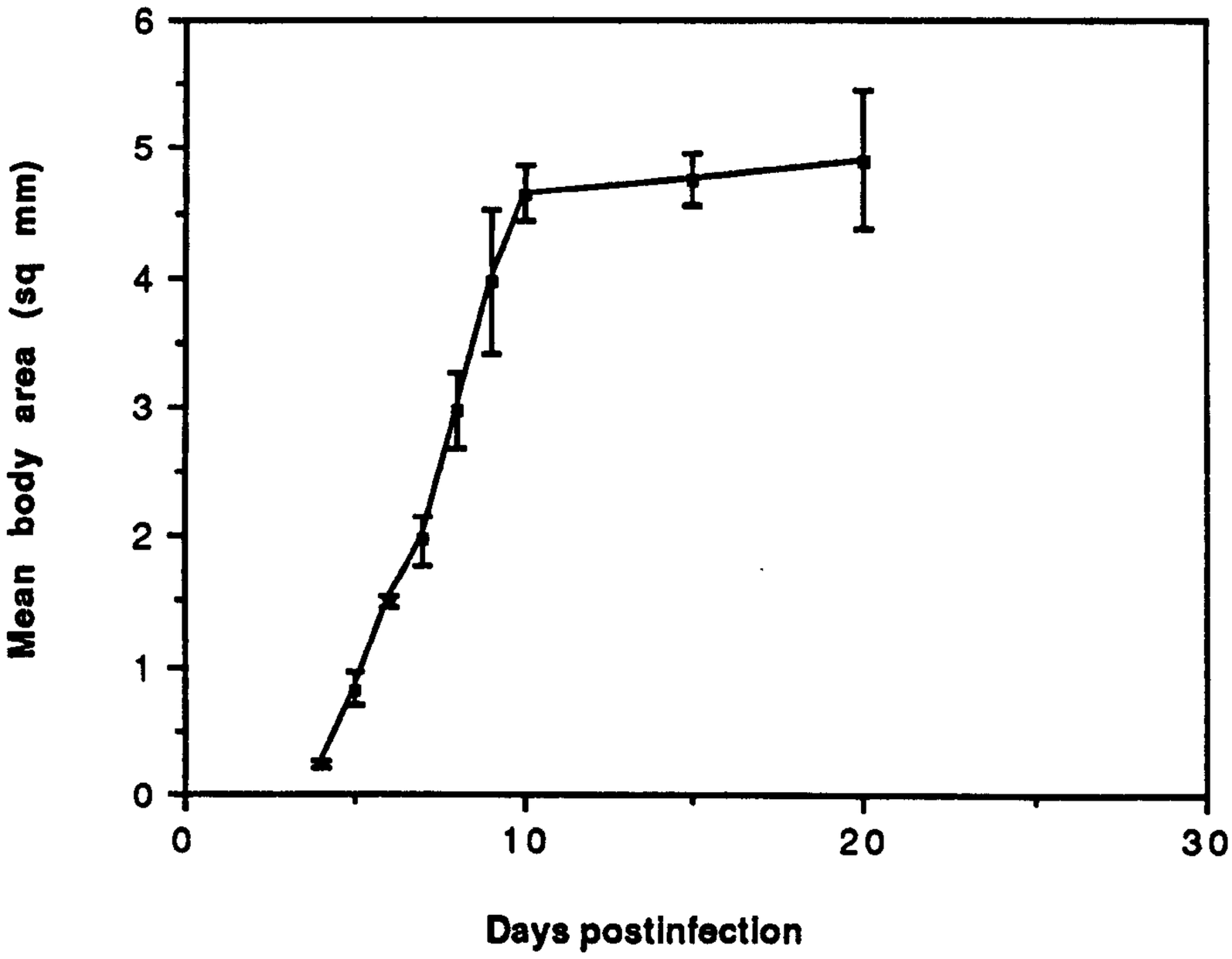

Mean body area (sq mm)
0
1
2
3
4
5
6
0
10
20
30
Days postinfection

Fig. 3.8

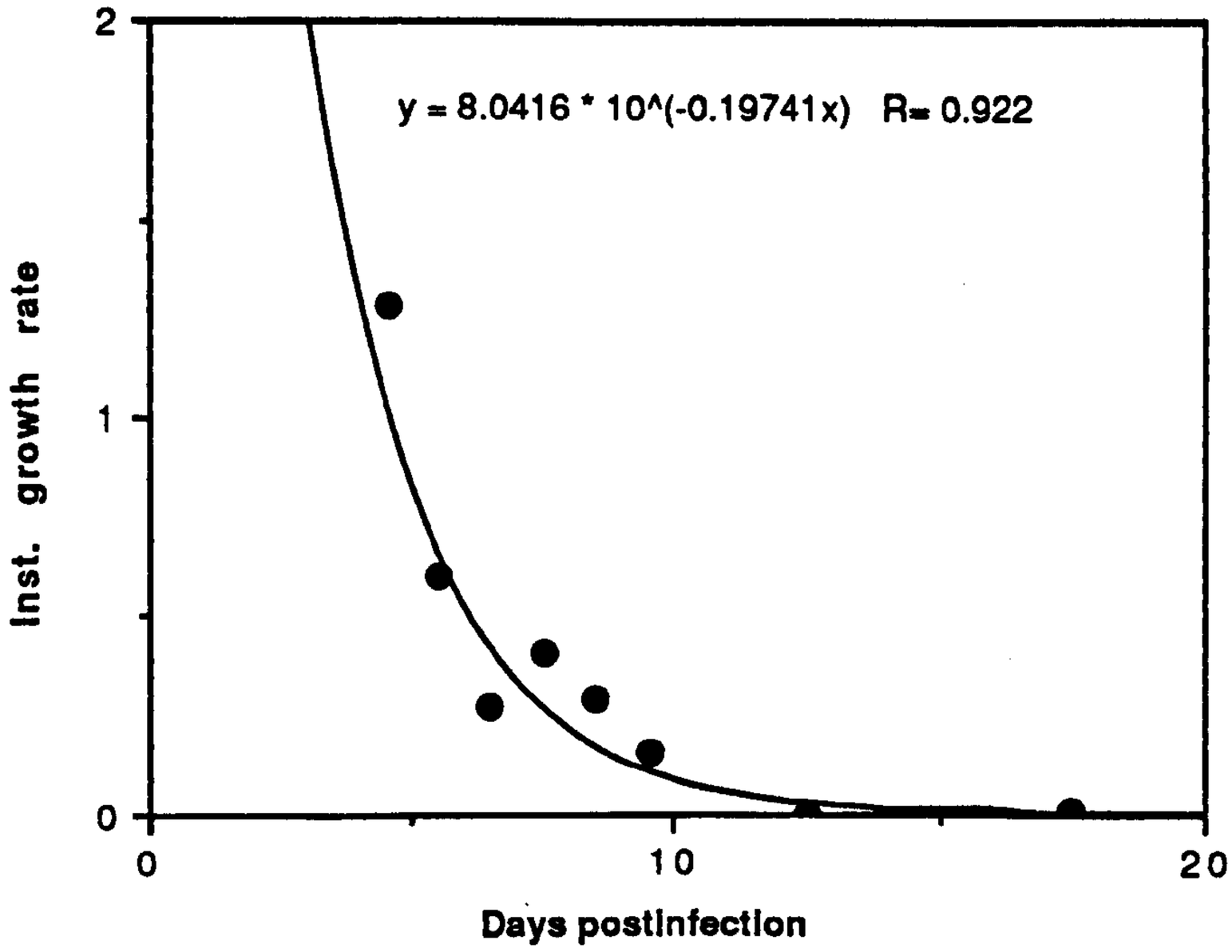

y = 8.0416 * 10^(-0.19741x) R= 0.922
Inst. growth rate
0
1
2
0
10
20
Days postinfection

Fig. 3.9 Mean vitelline gland area at successive days postinfection (data points include 95% confidence limits)

Fig. 3.10 Proportion of body area occupied by the vitelline glands

Fig. 3.9

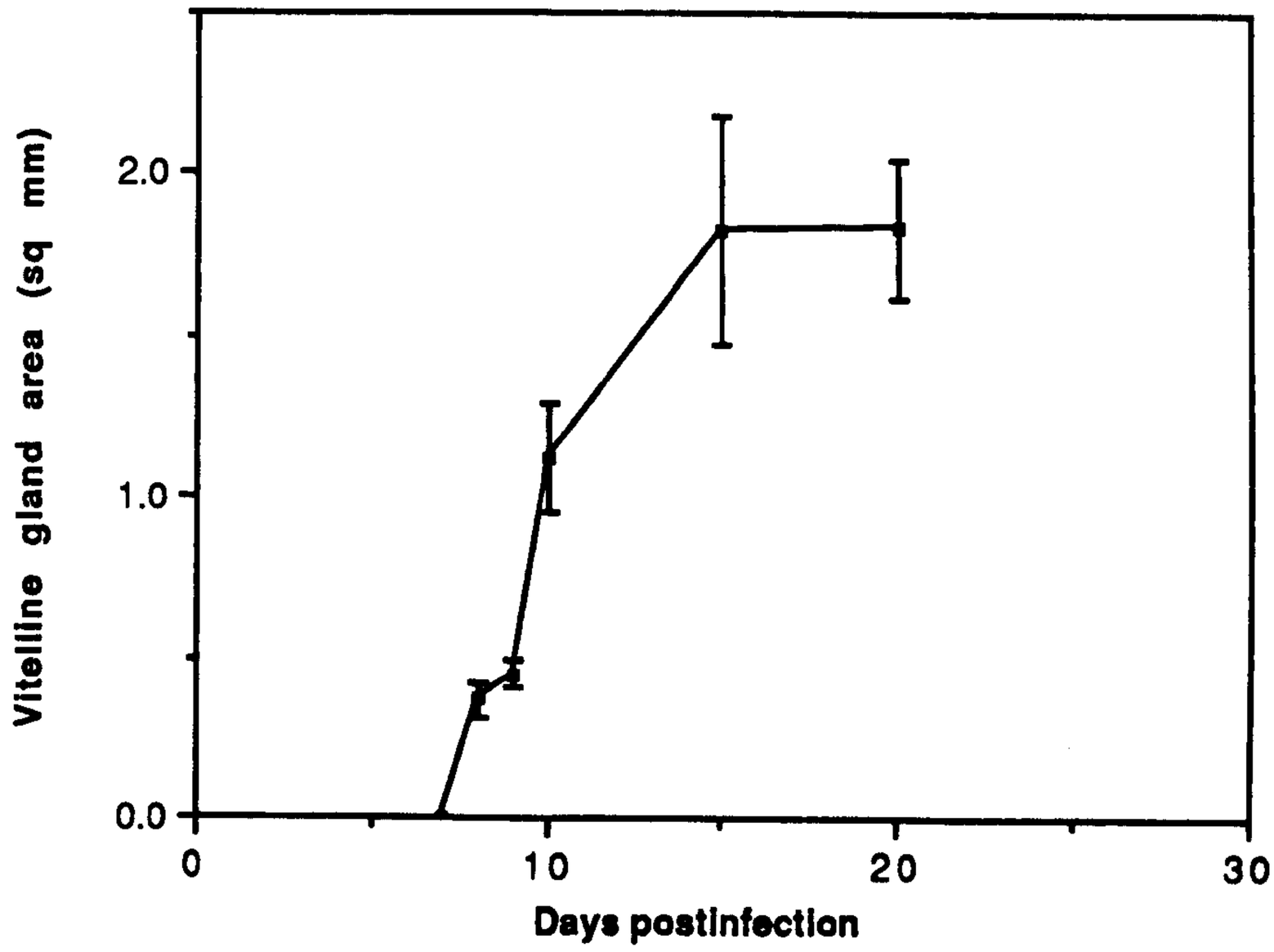


Fig. 3.10

which shows the changing vitelline gland area of *E.liei* with time from days 7 to 20 days postinfection. From 8 days onwards there is an increasing area occupied by the vitelline glands, from day 9 to day 15 there is a fourfold increase in proportional area (0.45 mm^2 to 1.83 mm^2), which begins to level out between days 15 and 20. Fig. 3.10 shows the percentage proportional area of the whole worm body occupied by the vitelline fields from 8 to 20 days postinfection. This shows values of about 12% on days 8 and 9, then rises to about 24% by day 10 and reaches an apparent maximum plateau level of around 38% by day 15.

3.3.2 Morphological development of *E. liei*

Investigations with Fast Red Salt B demonstrated the presence of putative phenolic egg shell precursors in the worms vitelline glands from day 8 of development onwards only. These brilliant and rapid diazotate reactions densely stain active vitelline glands which enabled the BBC digitiser technique to be used to calculate the area occupied by these glands as already described. Fig 3.11 shows an 8 day old worm of *E. liei* stained with Fast Red Salt B. The vitelline glands which stain a deep red, occurred in the lateral fields of the worm in the posterior 80% of the body and became more extensive with increasing age. The vitelline glands extend from the posterior end of the ventral sucker to the posterior extremity of the worm, coalescing ventrally and dorsally posterior to the testes. Slight reactivity was also seen in a linear tract connecting the two lateral fields posterior to the ovary on a number of occasions. This reactivity was presumed to be the staining of the contents within the vitelline ducts.

Fig. 3.11 An 8-day old worm of *E. liei* stained with Fast Red Salt B

Scale bar=1 mm

Key
V-vitelline gland

Whole mounts of worms stained with Borax carmine enabled the internal structures of the worms to be identified and the extent of development to be determined using light microscopy. Fig 3.12 shows worms aged at days 2, 3, 6, 8 and 10 postinfection stained with Borax carmine. As the worm ages it appears that the ventral sucker moves from a centrally located position to a more anterior one. This proportional change is seen more clearly in Fig. 3.13 as a diagrammatic representation of Fig. 3.12 in which it can be seen that the anterior section (anterior end of body to midpoint of ventral sucker) increases far less rapidly in size between days 2 and 10 than does the posterior section (posterior end of body to midpoint of ventral sucker). The two sections are of approximately equal length at day 2 whereas by day 10 the posterior section is approximately 4 times longer than the anterior section. The genital rudiments were first observed in some worms at 2 days postinfection. All the genital organs and the coiling of the uterus were recognizable by 4 days postinfection and the paired testes and ovary were completely distinguishable by day 6. Immature eggs and mature eggs were first observed in the proximal coils of the uterus in a number of 8 day old specimens and on every day thereafter postinfection. It would appear from this evidence and from the identification of putative phenolic egg shell precursors that the vitelline glands are morphologically and functionally complete by day 8. It can be seen from the stained whole mounts that as the worms age during the course of the infection the uterus becomes larger, ultimately becoming filled with eggs. The gonads are situated in the posterior region of the worm and the ovary appears oval in shape and occupies a median position while the

Fig. 3.12 (1-5)

Whole mount preparations of *E. liei* recovered from experimentally infected mice at :-

(1) 2 days postinfection

Scale bar= 0.1 mm

(2) 3 days postinfection

Scale bar= 0.1 mm

(3) 6 days postinfection

Scale bar= 1 mm

(4) 8 days postinfection

Scale bar= 1 mm

(5) 10 days postinfection

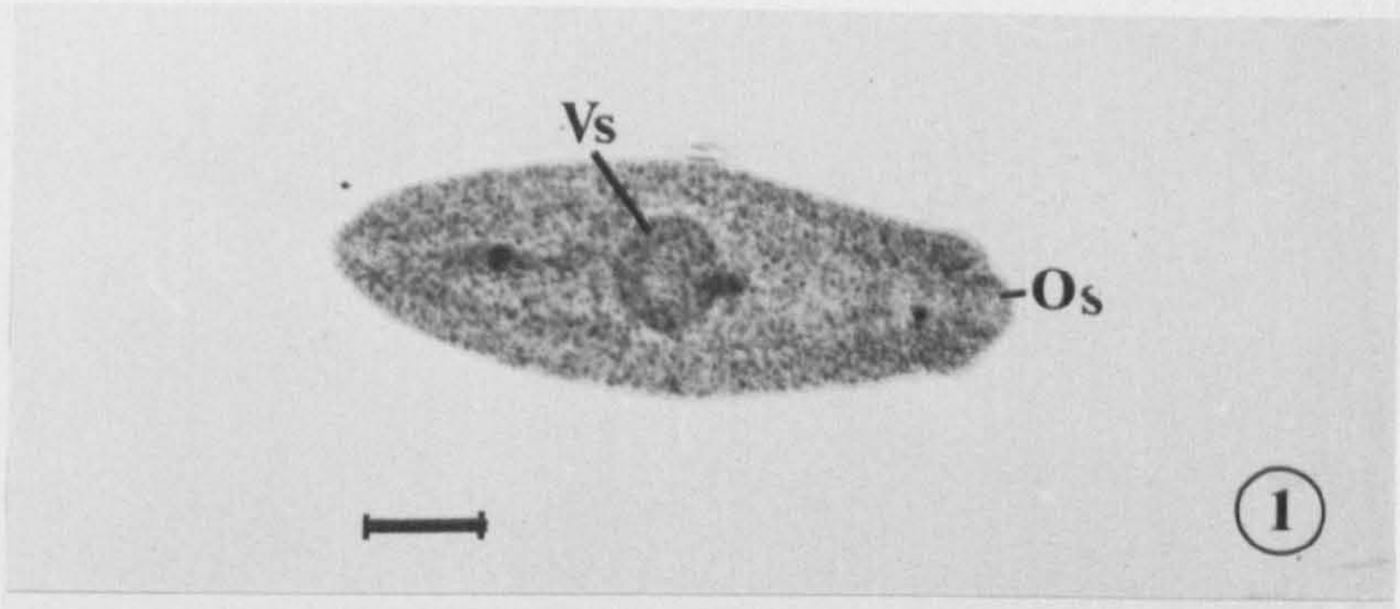
Scale bar= 1 mm

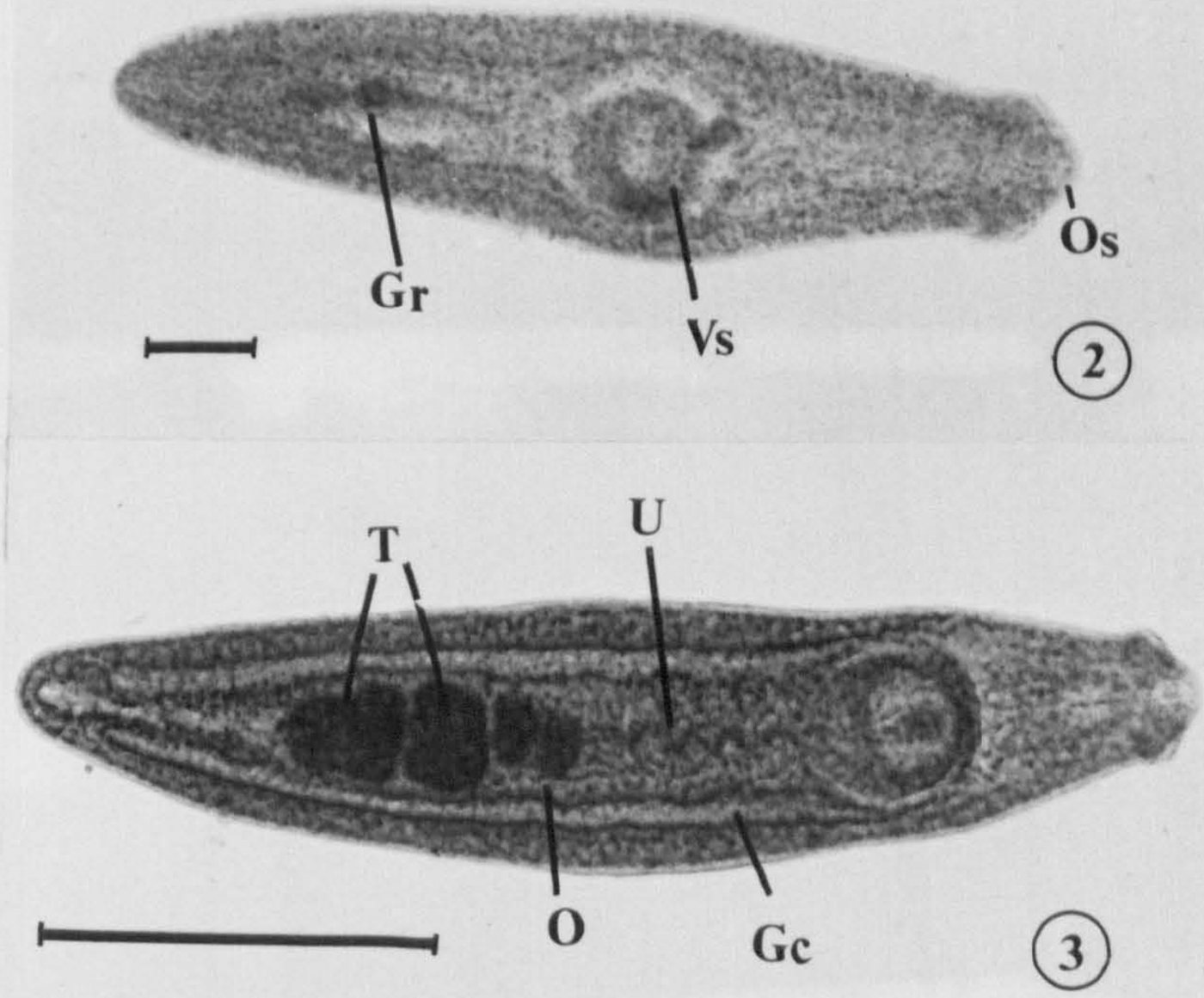
Key

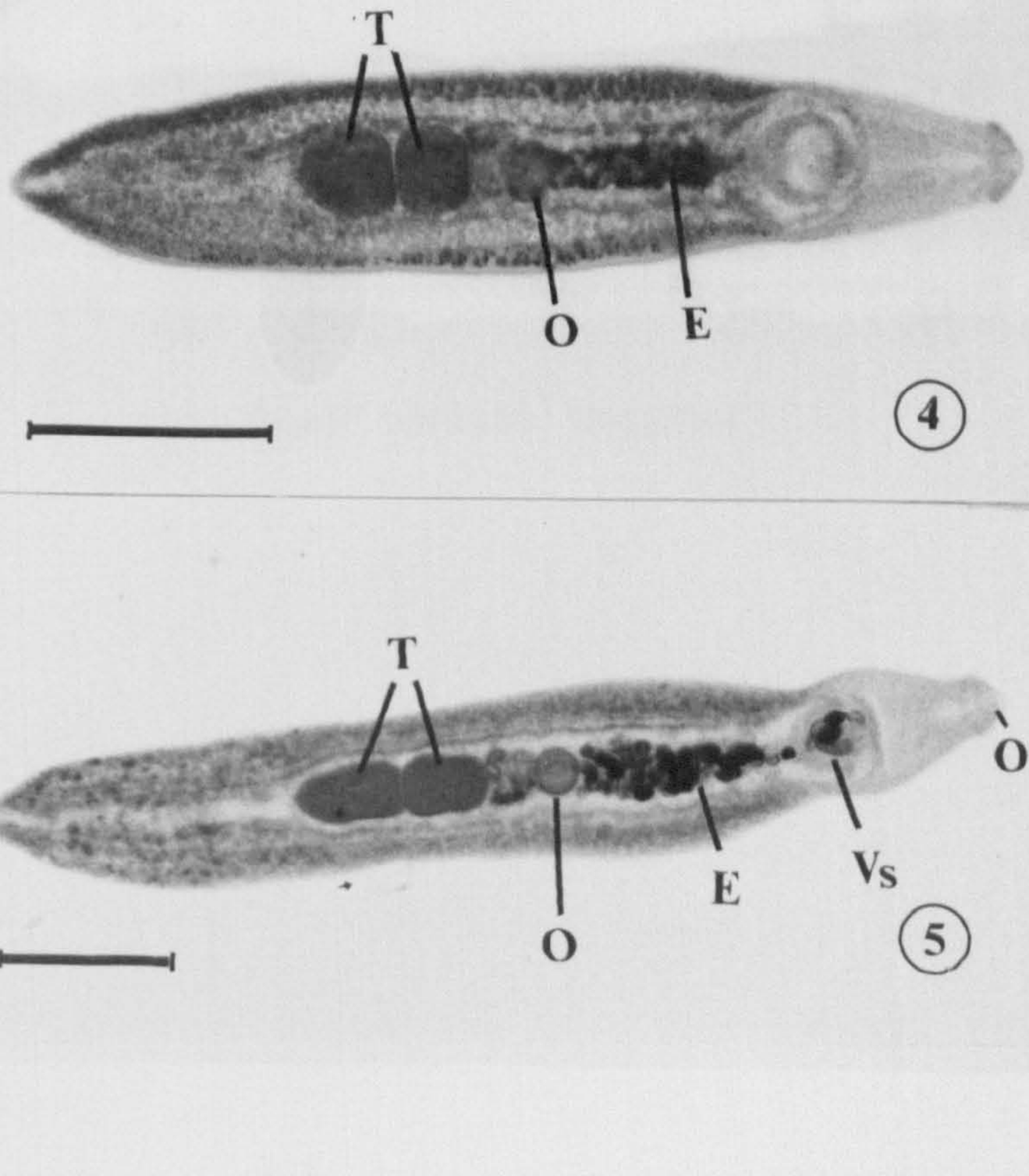
Os-oral sucker Gr-genital rudiments T-testes

Vs-ventral sucker U-coils of uterus O-ovary

Gc-gut caecum E-eggs (within uterus)


Vs
Os
1


Gr
Vs
Os
2
T
U
O
Gc
3


T
O
E
4
T
O
E
Vs
Os
5

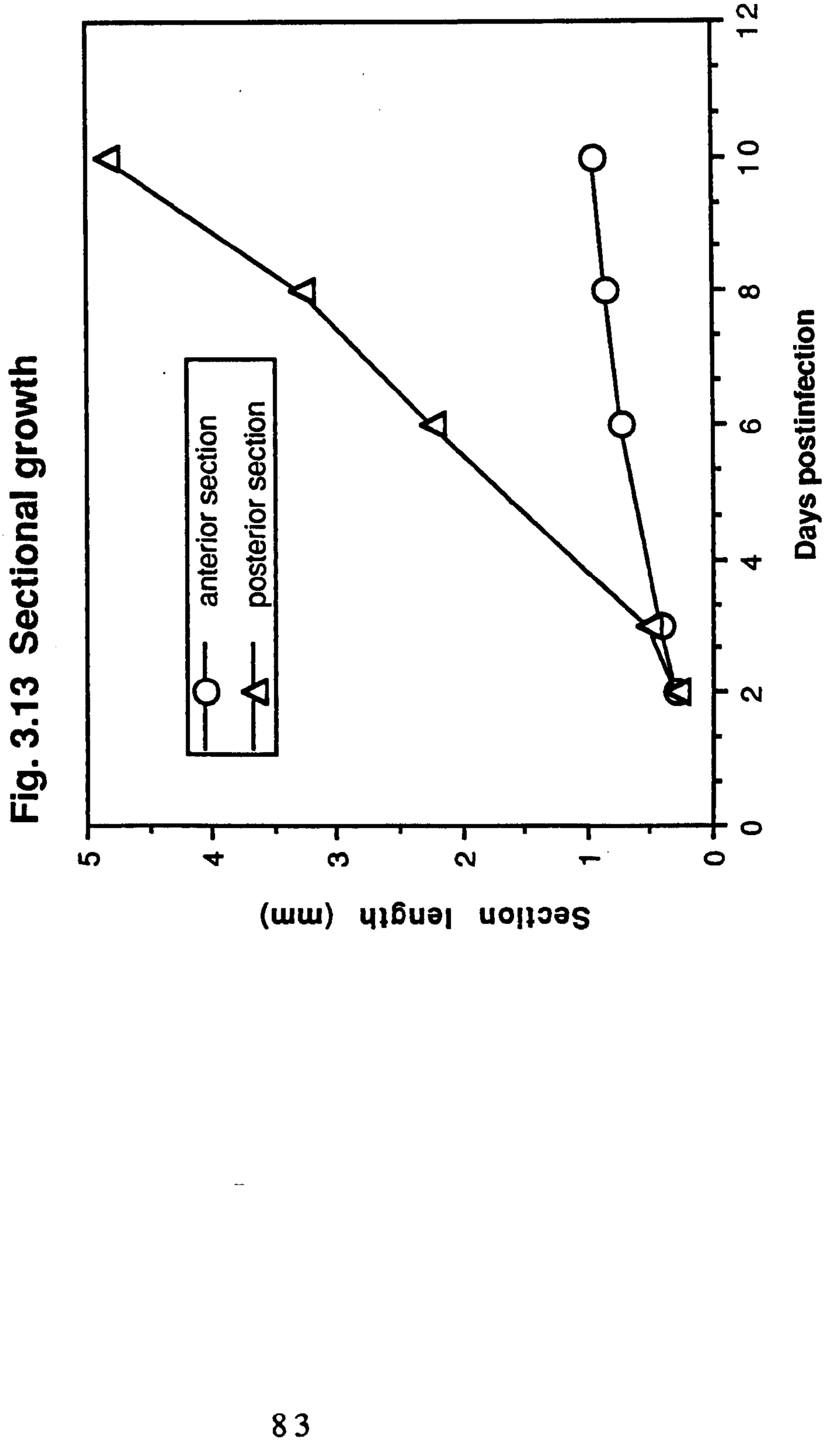


Fig. 3.13 Sectional growth
anterior section
posterior section
Section length (mm)
Days postinfection
0
1
2
3
4
5
0
2
4
6
8
10
12

testes are not as regular in their shape but are always present in tandem.

3.3.3 Scanning electron microscopy of *E.liei*

Mature worms at 8, 20 and 100 days postinfection were examined along with immature worms at 6 days postinfection. Observations of *E.liei* at these varying stages of development revealed the prominence of the ventral sucker, the oral sucker with its collar of spines and the tegumentary body spines. A whole adult worm of *E. liei* at 20 days postinfection is shown in Fig. 3.14 (1). Adult worms over 20 days postinfection have a very fleshy appearance when compared to that of an 8-day old worm and an immature 6-day old worm which both have a more streamlined appearance (Fig. 3.14 (2)). The anterior third of the parasite is shown in Fig.3.14 (2) and (3) from its ventral aspect. The oral sucker is surrounded by a horseshoe-shaped circumoral disc within which the collar spines are anchored (Fig. 3.14 (3) and (4)). A distinct ventral gap is present just below the oral sucker and anterior to the ventral sucker. This area appears slightly curved forming a valley-like pouch around the genital opening (Fig.3.14 (2) and (3)). The oral sucker, which surrounds the mouth appears smooth in all of the stages studied (Fig. 3.14 (4)). The collar spines which partially surround the oral sucker are small and peg-like with bluntly ending tips and on most occasions appear to be partially retracted (Fig. 3.14 (4)). A distinct group of corner spines are evident at the edge of the circumoral disc on each side of the ventral gap and it can be seen that some of these spines are partially extended (Fig.3.14 (5)). The collar spines appear to be able to be withdrawn into pockets in the tegument of the circumoral disc (Fig. 3.14 (3).

Fig. 3.14 (1-6)

Scanning electron microscopy of *E. liei*

(1) Adult worm 20 days postinfection

Scale bar=100 μm

(2) Anterior ventral view 8 days postinfection

Scale bar=100 μm

(3) Genital opening 20 days postinfection

Scale bar= 100 μm

(4) Oral sucker and circumoral disc 8 days postinfection

Scale bar= 100 μm

(5) Collar spines 8 days postinfection

Scale bar= 25 μm

(6) Ventral sucker 6 days postinfection

Scale bar= 50 μm

Key

Os-oral sucker Vs-ventral sucker Cs-collar spines

Vp-ventral pouch Go-genital opening Cd-circumoral disc

Ts-tegumental spines

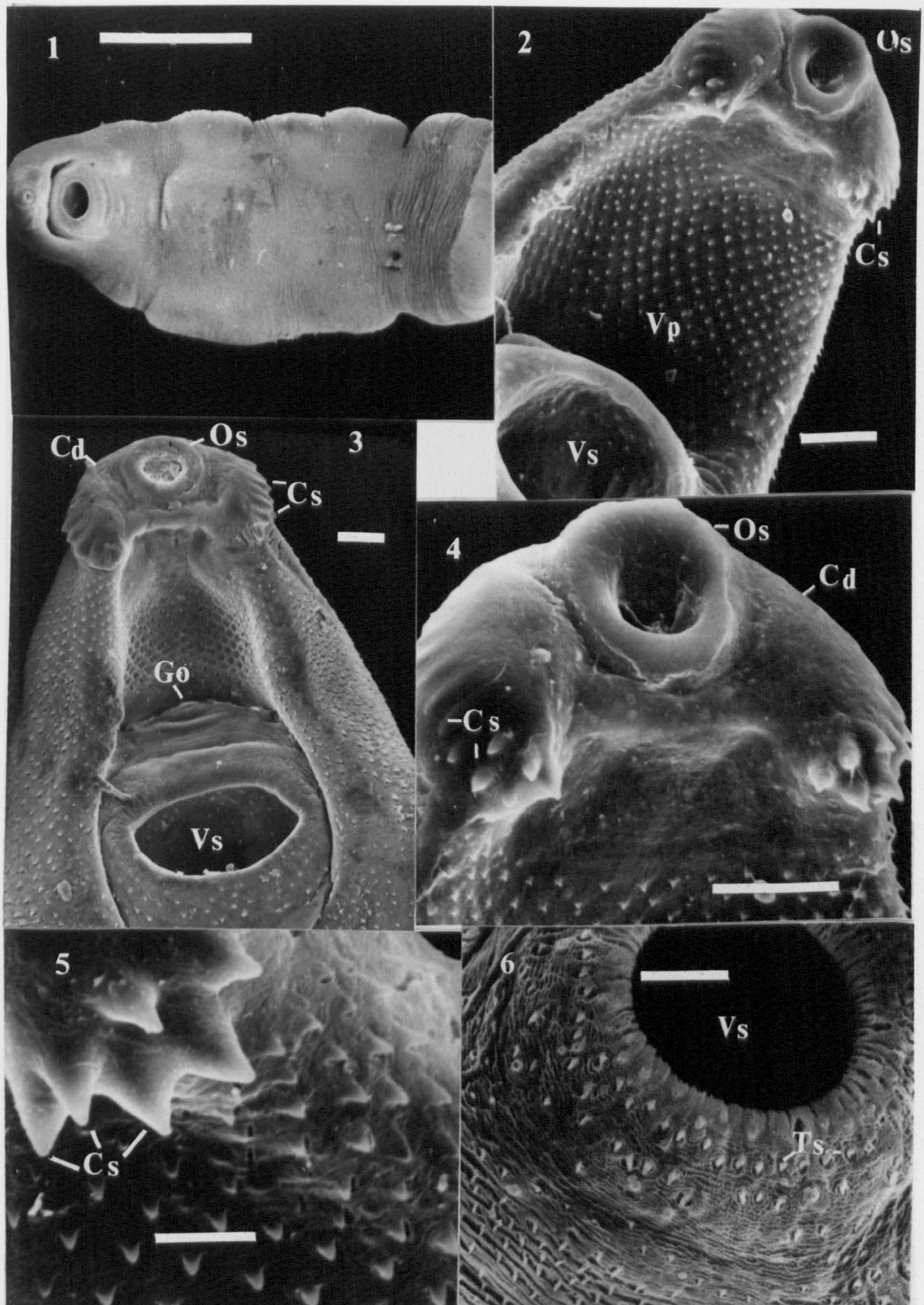

1
2
Os
Cs
Vp
Vs
3
Cd
Os
Cs
Go
Vs
4
Os
Cd
Cs
5
Cs
6
Vs
Ts

Fig. 3.14 (7-14)

Scanning electron microscopy of *E. liei* (cont.)

(7) Vental sucker 8 days postinfection

Scale bar= 100 μm

(8) Cirrus 100 days postinfection

Scale bar= 100 μm

(9) Ventral tegumental spines 20 days postinfection

Scale bar= 10 μm

(10) Ventral tegumental spines 8 days postinfection

Scale bar= 10 μm

(11) Multipointed tegumental spines 20 days postinfection

Scale bar= 10 μm

(12) Anterior dorsal view 20 days postinfection

Scale bar=100 μm

(13) Sensory papillae 20 days postinfection

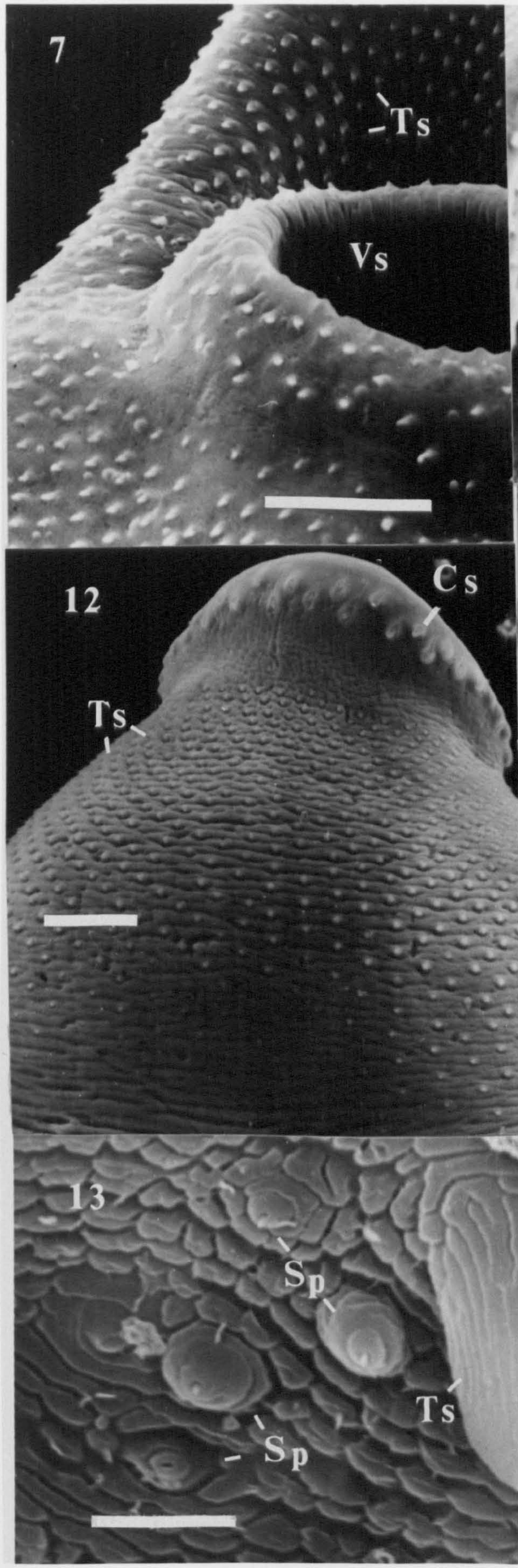
Scale bar= 5 μm

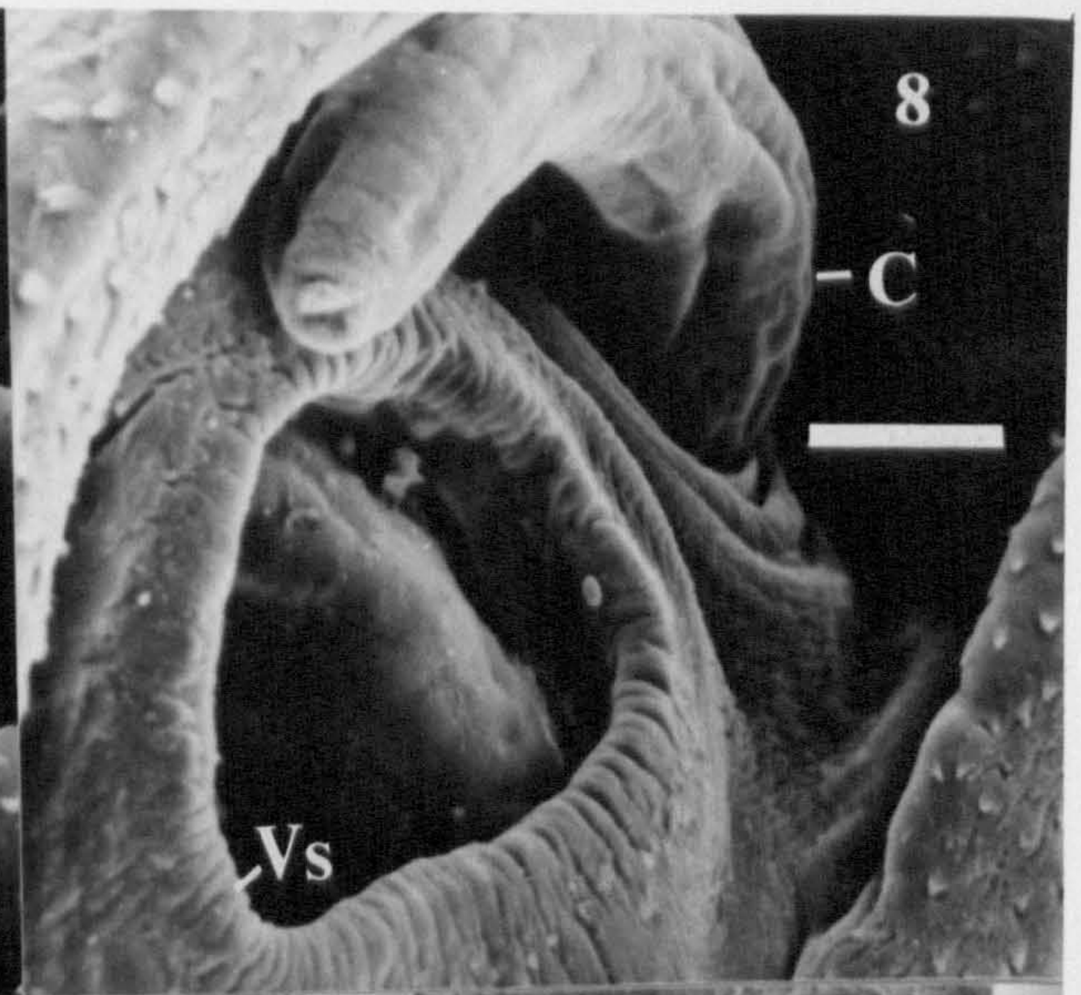
(14) Excretory pore 20 days postinfection

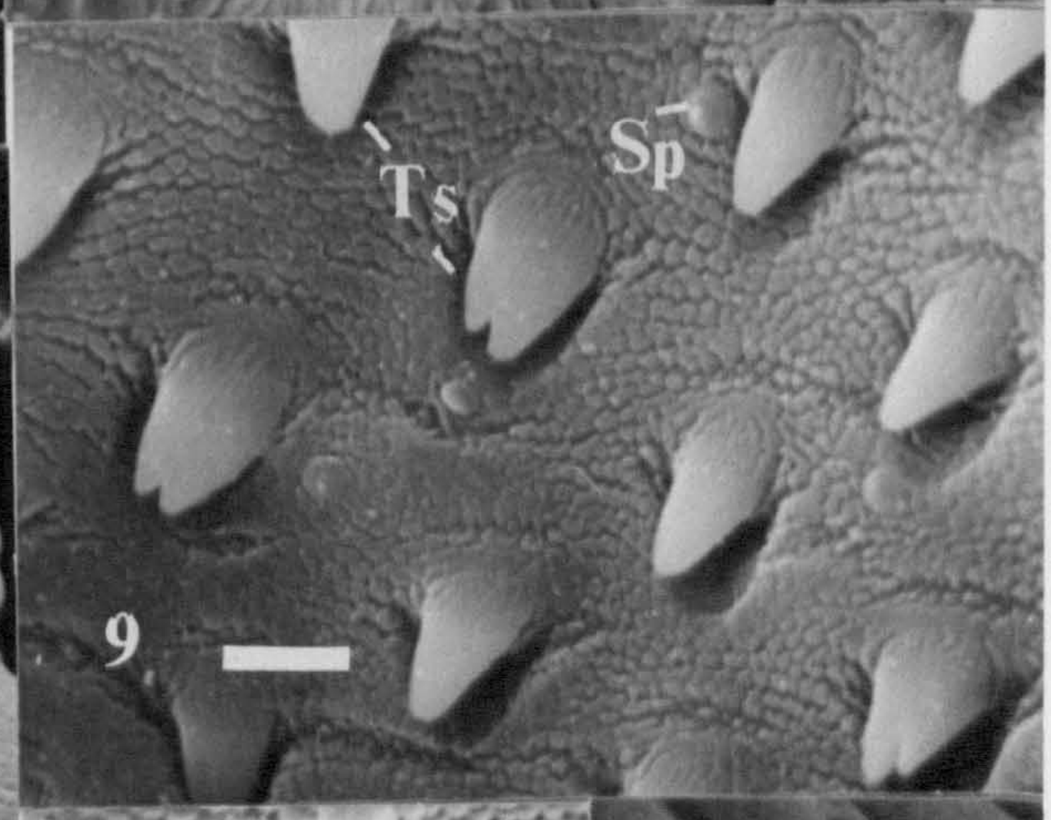
Scale bar= 25 μm

Key

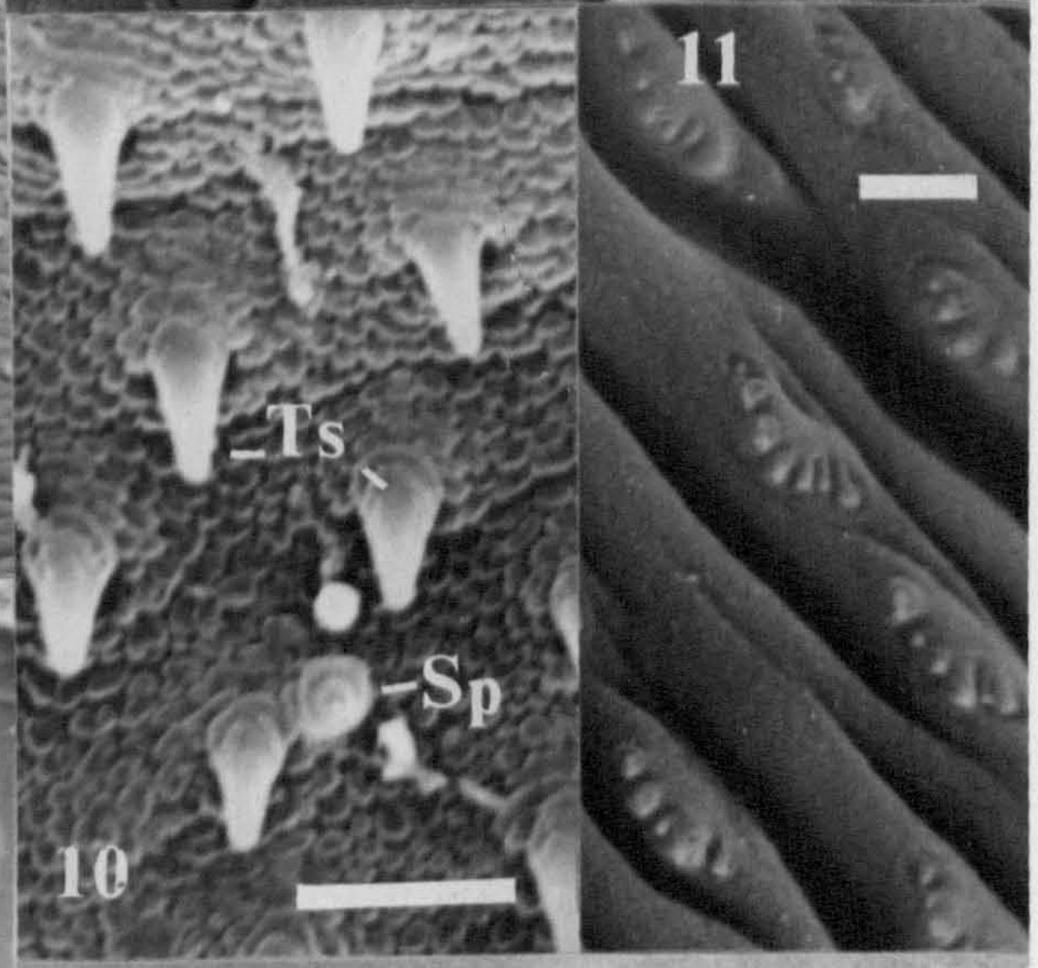
C-cirrus Sp-sensory papillae Ep-excretory pore


7
Ts
Vs

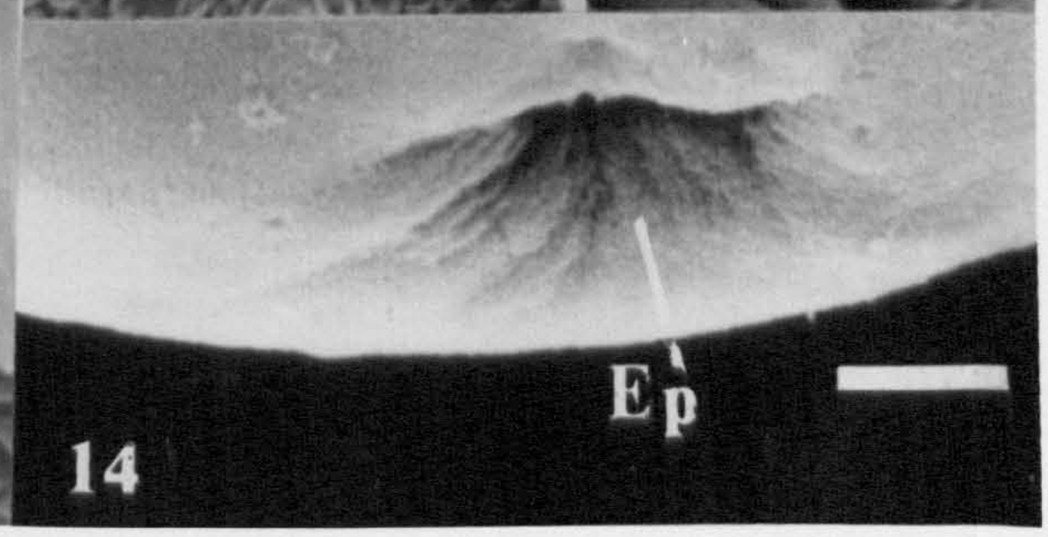

8
C
Vs


Ts
Sp
9

12
Cs
Ts


11
Ts
Sp
10

13
Sp
Sp
Ts


Ep
14

and (4)). The ventral sucker of the worms studied lies in the anterior third of the body Fig. 3.14 (3), (6) and (7). The general tegument of the sucker is smooth at its innermost margin but contains scattered flattened spines surrounding this area and a number of tegumental spines on its outermost surface. Directly anterior to the ventral sucker is located the genital opening (Fig. 3.14 (3)) from which extends the highly protrusible cirrus shown in a 100 day old worm (Fig. 3.14 (8)). The elongate cirrus is smooth and cylindrical in structure and posesses an undivided tip at the end of which opens a simple terminal pore. The cirrus tegument appears to be aspinose and its surface bears no resemblance to that of the body tegument.

In all worms observed the abundance of tegumentary spines on the ventral surface of the worms are a striking feature (Fig.3.14 (9), (10) and (11)). These conspicuous spines, which have pointed tips, become larger and more defined as the worms age. They occur as regularly arranged rows and are prominent in the anterior three quarters of the ventral surface (Fig. 3.14 (9) and (10)) and appear to decrease very slighty in number in the posterior quarter of the ventral surface where they appear to take the form of multipointed spines (Fig. 3.14 (11)) in mature worms. The dorsal surface of the worms reveals a contrasting picture. Spines are present only in the anterior quarter of the worms body and are less pointed and the remaining surface appears as a fleshy ridged region devoid of any spines (Fig. 3.14 (12)). The sensory stuctures present in these worms were the uniciliate domed papillae shown in Fig. 3.14 (13). These sensory papillae occured singularly or in groups numbering 2 to 3. They were very prominent on the ventral surface of the worm

in the anterior third of the body. The majority of these papillae appeared to be located within the ventral pouch. These structures were absent from the posterior third of the worms ventral surface. The posterior region of the worm terminates as a bluntly rounded point where a prominent excretory pore is located (Fig. 3.14 (14)).

3.4 Discussion

Infectivity

Previous growth studies involving *E. liei* in chicks, which have commented on the growth of this intestinal parasite over five weeks showed that all chicks became infected and the mean percentage recovery of worms was 21.6% (Fried and Emili, 1987). A later study by Fried, Donovick and Emili (1988) observed a 97% prevalence of chicks infected with 100 metacercarial cysts with a worm recovery of 28% at 28 days postinfection. Interestingly, beyond 3 weeks the worm population declined markedly, with chicks on day 35 containing no worms. Franco, Huffman and Fried (1986) using *Mesocricetus auratus* as a host, and doses of 100 metacercarial cysts of *E. revolutum* showed that 100% of the hamsters became infected and worm recovery averaged 38%. Fried's (1984) earlier study of infections of domestic chicks with *E. revolutum* also noted that all chicks became infected and there was a mean worm recovery rate of 25% from 1 to 44 days after exposure. Worms were recovered from the ileum, caeca, rectum-cloaca and the bursa of Fabricius. A later comparative study by Hosier and Fried (1986) of infections of *E. revolutum* in Swiss Webster (SW) and ICR mice each fed 25 metacercarial cysts showed that at 2 weeks postinfection 75% of the worms were recovered from SW mice, but only 22% from ICR mice. They also found that no

worms were recovered from ICR mice at three weeks nor from SW mice at four weeks postinfection.

The study of Mohandas and Nadakal (1978) of *E. malayanum* included a report on development of this echinostome in white rats from 18 hours to 13 days postinfection. After infecting rats with 10 metacercarial cysts of this worm they found that the percentage establishment of worms over this period was apparently 50-70% and in the remaining days 86-94%. They stressed that the former establishment value may have been artificially low because of the difficulties encountered in recovering worms in infections of short duration from 18 hours to 2 days postinfection. They explained that worms were small, transparent and difficult to distinguish when mixed with intestinal contents and therefore made identification difficult. In the present study with the experimental infection protocol (that is, 25 or 50 metacercarial cysts per mouse) a 100% infection prevalance was achieved. As can be seen in Fig. 3.1, the apparent percentage establishment of worms rose to values between 44%-65% after about 5 days postinfection. The most likely explanation for this rise in apparent establishment between day 1 and day 5 is the same worm identification problem noted by Mohandas and Nadakal (1978). At this early phase of infection it seems clear that all the small transparent worms were not being recovered from the gut. Fig. 3.1 also makes it clear that after an initial establishment success of between 44% and 65% no significant mortality in worms appears to occur up to 100 days postinfection. In this host *E. liei* is long-lived.

Growth and development patterns

Growth in length and width of *E. liei* are in direct proportion to one another over 100 days but over the first 20 days it is apparent that width increases more rapidly than length. Similarly, the ventral sucker increases more rapidly in size than the oral sucker over 100 days. Dawes (1962) in his study on the growth of *F. hepatica* in mice showed that greater increments were added to the length rather than to the width of the worm and to the diameter of the ventral sucker than to the oral sucker. Saito (1984) observed that the growth in length and suckers of *E. hortense* in Wistar rats followed a linear pattern and stated that the growth curves of the testes, ovary and cirrus sac were sigmoid-shaped.

The growth rates and development patterns have been determined for a number of digenetic trematodes. Fried (1962) looked at the growth rate of *Philophthalmus* sp. in the eyes of chicks and commented on the fact that the pattern of growth, expressed as length against successive times postinfection, adopted the shape of a sigmoid curve. This classical S -shaped curve is characteristic of many digenean growth patterns identified by workers such as Senger (1954), Nollen (1971) and Saito (1984). Nollen divided similar sigmoid-shaped curves for length increases into three phases characteristic of the growth of free living organisms, the lag phase, the exponential phase or log phase and the stationary phase. This characteristic growth pattern is exhibited by *E. liei* with respect to growth in both length and width in the semi-log plot expressed in Fig. 3.3 and its growth in area (Fig. 3.7). Growth increments are at their greatest between days 4 and 10 postinfection for all these dimensions. From day 20 onwards

growth is similar to the stationary phase observed by Nollen. Fried, Donovick and Emili (1988) after infecting chicks with 100 metacercarial cysts of *E. liei* found that mean worm measurements for length and width had the following values. By 7 days postinfection worms measured 2.97 x 0.54 mm for length and width respectively, by day 14, 4.90 x 0.96 mm and by 21 days 5.60 x 1.12 mm. Fried and Emili (1987) infected day old chicks with 100 metacercarial cysts of *E. liei* and estimated the area of the worms (adopting the Berntzen and Macy, 1969 model) that gave a first order estimate of the projected body area from day 7 to day 28 post-exposure. They found that body area of *E. liei* increased from 0.9 mm^2 on day 7 to 3.6 mm^2 on day 28. On days 14 and 21, body area was calculated as 2.8 mm^2 and 3.3 mm^2 respectively. It would appear from a direct comparison of these results that *E. liei* is larger on the respective days postinfection with respect to both area and length increases, in Swiss T.O. mice than in chicks.

The localization of putative phenolic substances in the vitelline glands using Fast Red Salt B suggests that the vitelline glands of *E. liei* contain elements of a sclerotin-forming tanning system which is fully functional at 8 days postinfection. The egg shells of most digeneans, monogeneans and many tapeworms (except those of the cylophyllideans) show tanning or sclerotization, a hardening process involving phenols, proteins and phenolase enzymes (Breckenridge and Nathanael, 1988). The diazo-coupling technique as a means for identification for the occurrence of phenolic substances has been discussed by Bell and Smyth (1958), Johri and

Smyth (1956) and Breckenridge and Nathanael (1988). Johri and Smyth proposed the use of this salt as a means to study certain aspects of helminth morphology because of its ability to link readily with phenolic materials giving characteristic colours. Hence the earliest appearance and subsequent fate of the egg shell precursors may be detected by means of this method. These methods are based on the fact that the vitelline glands of most adult digeneans are rich in proteins and phenols. These phenolic egg shell materials are released during egg shell formation and combine with proteins to form a quinone-tanned egg shell (Johri and Smyth, 1956; Smyth and Halton, 1986).

Fried, Donovick and Emili (1988) explained that in *E. liei* grown in chicks, the testes became distinct in stained worms by days 2 or 3, coiling of the uterus was seen by day 5 and vitelline glands were present on day 6. They also noted ovigerous worms at 7 days postinfection. Mohandas and Nadakal (1978) divided the developmental process of *E. malayanum* into four stages which they based on the seven stages in the development of strigeids listed by Bell and Smyth (1958). These four stages were as follows :

Stage 1. Organogeny, which incorporated days 1 to 4 postinfection and involved the appearance of the testes and ovary at day 4 and the genital pore by day 5.

Stage 2. Vitellogenesis, completed during days 6 and 7, with the appearance of lateral vitelline cells while the uterus becomes conspicuous.

Stage 3. Formation of Mehlis gland complex and cirrus sac from days 8 to 10.

Stage 4. Oviposition, during days 11 to 13 and involving the appearance of eggs in the proximal coils of the uterus on day 11, while by 13 days the uterus was completely filled with eggs.

It is apparent from observations of *E. liei* grown in mice and those of Fried and his co-workers observing its growth in chicks that the different stages of development and growth of *E. malayanum* are applicable to the growth of *E. liei* but with a slightly differing time scale as revealed by the stained whole mounts. The first appearance of the rudiments of the ovary and testes is within a similar time frame. The vitelline glands were complete and apparently functional at 8 days postinfection for *E. liei* in mice compared with days 6-7 for *E. malayanum*, but the eggs appeared much earlier in *E. liei* at 8 days postinfection in mice and 7 days postinfection in chicks while their appearance in *E. malayanum* was noted at 11 days postinfection.

The rate of growth and differentiation of digeneans are genetically determined in the main (Halton and Smyth, 1986) but it is evident from the numerous studies on parasite growth that body developmental patterns may differ markedly in different hosts or in different strains of the same host. Evans (1983) after observing a similar pattern of establishment and survival of *E. recurvatum* in Khaki Campbell ducklings to Senger's (1954) experiment with *E. recurvatum* in the fowl, *Gallus gallus* commented that parasites which demonstrate broad host specificity often show characteristic rates of establishment, development and survival in each of the host species utilized. Kinsella (1971) looked at growth and development of the notocotylid, *Quinqueserialis quinqueserialis* in three rodent hosts *Microtus montanus*, *Microtus pennsylvanicus*

and *Ondatra zibethicus* and noted growth varied between hosts. He constructed growth curves of length and width against age in days and showed that growth expressed as an increase in length was greater in *M. montanus*, followed by *M. pennsylvanicus* and slowest in *O. zibethicus*.

In vitro cultivation of worms has also revealed major differences in growth patterns when compared with those same worms grown *in vivo*. Davies and Smyth (1978) found that somatic development of newly excysted juveniles of *F. hepatica* grown *in vitro* was similar to that of worms recovered from the liver of a mouse 11 days postinfection but noted no corresponding development of the genital rudiment of worms grown *in vitro*. Fried, Fiene and Felter (1980) observed that the mean body area of *L. constantiae* recovered from chicks was 2-3 times greater than worms grown on the chorio-allantois of chick embryos in culture. The complete morphological development of *E. liei* in the mouse does reveal the capability of this parasite to survive within a laboratory host. Whether laboratory growth rates are comparable with those exhibited by *E. liei* in its natural hosts in the natural environment cannot be decided until studies involving its Egyptian definitive hosts are carried out.

Scanning electron microscopy observations.

The surface topography of mature and immature worms of *E. liei* revealed in this study show a fundamental similarity to each other and to those of other adult echinostomes previously studied by SEM. *E. liei* posesses 37-collar spines as does *E. revolutum* while *E. malayanum* contains 44, *I. melis*, 27 and *M. denticulatus*, 22. In all

of these cases the probable function of these collar spines is similar. The retractable collar spines which partially surround the oral sucker may be involved in the attachment of the worms to the host mucosa and thus help to maintain the position of the worm in the intestine of its host and to hold the oral sucker close to the mucosa for feeding. Such attachment-adaptations would be beneficial during the worm population's ontogenetic migrations (see Chapter 5) against the intestinal peristaltic flow and may be directly or indirectly related to the pathology associated with *Echinostoma* infection (see Chapter 6) which includes erosion of the villous structure. Thulin (1980) has pointed out that the adjustable spines of *Aporocotyle simplex* may provide increased efficiency of attachment to the host. McDowell and James (1988) have recently suggested a feeding role for the circum-oral spines of the gut parasitic acanthosome *Timoniella imbutiforme* because of their capacity to abrade the hosts gut wall.

The oral and ventral suckers of *E. liei* at all the ages studied showed marked similarities to those of *E. malayanum* (Tesana *et al.* 1987). Koie (1987) noted plate-like spines round the edges of both suckers in 4-day old worms of *M. denticulatus* while in mature 10 day old worms these spines were not present on the ventral sucker while those of the oral sucker persist. In both *E. revolutum* and *I. melis* the ventral sucker is aspinose but the rim of the ventral sucker in both cases was surrounded by numerous papillae (Smales and Blankespoor, 1984). McCarthy (1989) in a SEM study of *Echinoparyphium recurvatum* similarly observed papillae only on the outer lip of the ventral sucker ring. Baake (1976) used SEM to study *Leucochloridium* sp. and observed the absence of spines on

the suckers and suggested that this may indicate the necessity for a smooth seal against the host mucosa.

The distribution of the body tegumental spines in *E. liei* follows a similar pattern to that of *E. malayanum, I. melis* and *E. revolutum* but differs from *M. denticulatus* where Koie (1987) observed no spines on the posterior three quarters of the ventral worm surface in worms recovered from chickens. In the SEM study carried out by Smales and Blankespoor (1984), in which they recovered adult worms of *E. revolutum* and *I. melis* from musk rats (*Ondatra zibethicus)* and wild mink (*Mustela vison)* respectively, they noted that the tegument of the body surface of the worms had a cobblestone-like appearance with interspersed pits. In the anterior three quarters of the ventral surface the spines of *E. revolutum* had a scale like appearance while in *I. melis* tegumentary spines were rounded and blunt. Fried and Fujino (1984) described the tegument of *E. revolutum* recovered from chick embryos and domestic chicks and described the tegument in 7 day old worms as being granular in both cases. Tesana *et al.* (1988) noted that the body spines of *E. malayanum* recovered from rats were scale-like and had a triangular shaped appearance with blunt tips. The multi-pointed tips reported in this study for *E. liei* appear to be unique to *E. liei* amongst the species so far observed and are comparable to those spines reported for *F. hepatica* (Bennett, 1975). Spines of the ventral surface of *E. liei* which were in a posterior position appeared multi-pointed but these were only observed in mature worms. Bennett (1975) demonstrated in his work on spine development in *F. hepatica* that single-pointed spines metamorphosed to multi-pointed spines with divisions in the tips

and suggested a possible locomotory/feeding role for these spines and also observed marked changes in tegumentary spines during postmetacercarial development in the mouse. All spines in *E. liei* appeared to point in a posterior direction, that is, towards the excretory pore. It is evident that there is marked variation between the shape of the tegumental body spines in the different *Echinostoma* species even though the spine distribution is very much the same in the majority of the echinostomes studied. The dorsal side of *E. liei* appears to play no major functional part in the maintenance of the position of the worm, since it appears the worms are attached to the mucosa by means of their ventral sucker. The only spines needed on the dorsal surface would be those in the anterior position, as observed in *E. liei*, since once embedded into the mucosa this region is probably curled around and becomes into contact with the mucosa. The anterior, lateral and ventral sides have the functional importance of being the area of the tegument which can be drawn (by the relatively strong developed suckers) into close contact with the host intestinal epithelium. These particular areas of the worms surface are those which are heavily armed with numerous tegumentary spines. Baake (1976) suggested that the smooth surface of the dorsal side is an advantage since it gives minimum friction against the passing stools. Beaver (1937) noted using light microscopy techniques, that tegumentary spines in a posterior position in *E. revolutum* tended to disappear in warm blooded hosts.

The sensory papillae observed in *E. liei* were similar to those already described for the other echinostomes studied. These unicilate sensory papillae are commonly located in general on the

anterior ventral surface of the body in *M. denticulatius* (Koie, 1987), *E. malayanum* (Tesana *et al.*, 1987) and *E. revolutum* and *I. melis* (Smales and Blankespoor, 1984). These studies do not specifically mention the ventral pouch where, in *E. liei,* numerous clusters of sensory papillae were observed. This type of sensory papilla was also found on the ventral sucker of *M. denticulatus* (Koie, 1987). The functions of such papillae have been speculated upon by Page, Nadakavukaren and Huizinga (1980) in the case of *Ribieroia marini* and Hoole and Mitchell (1981) in the case of *G. vitellobia.* These workers have explained that these papillae may function as mechano- and stretch-receptors controlling the contact attachment and release of the ventral sucker as the parasite moves about within its intestinal habitat.

The present study has revealed that the cirrus of *E. liei* contains no spines like those of *E. revolutum* and *I. melis,* although Smales and Blankespoor (1984) did observe that there was a difference in cirrus morphology of the latter two species. In *E. revolutum* they found the cirrus to be elongate with a bilobed tip with papillae on the tip surface while in *I. melis* it had a cylindrical appearance with scattered papillae over its surface. McCarthy (1989) described an aspinose cirrus surface in *Echinoparyphium recurvatum* which posessed a single tip and sessile aciliate papillae in the mid-region of the cirrus. The cirrus in both *E. malayanum* (1987) and *M. denticulatus* (Koie, 1987) were not observed.

This study has revealed some outstanding features of the 37-spined echinostome *E. liei* providing a basis for future morphological studies. In particular SEM observations on the

remaining disputed 37-spined African echinostomes may provide some useful morphological information for determining unequivocally patterns of relatedness among the African species.

CHAPTER 4

THE ADULT HERMAPHRODITIC REPRODUCTIVE SYSTEM OF *ECHINOSTOMA LIEI* WITH ULTRASTRUCTURAL OBSERVATIONS ON VITELLOGENESIS AND SPERMATOGENESIS

4.1 Introduction

The general organisation of the adult hermaphroditic system of digeneans has been outlined and described by Smyth and Halton (1983). In general, copulation in digeneans occurs by the insertion of the cirrus of one worm into the terminal portion of the uterus of another via the genital pore or in some instances by the insertion of the cirrus into the opening of the Laurer's canal (Chappell, 1980; Smyth and Halton, 1983). Fertilisation in all hermaphroditic digeneans is assumed to follow the basic mecahnism outlined by both Chappell (1980) and Smyth and Halton (1983). In this mechanism ova, which are produced in the ovary, are released periodically into the oviduct. At the same time, vitelline cells are released from the vitelline glands as are spermatozoa from the seminal receptacle. Fertilisation of the egg takes place within the ootype. The vitelline cells release their stored globules of egg shell precursor which form the egg shell.

The adult hermaphroditic reproductive system of *E. liei* was first described by Jeyarasasingam, Heyneman, Lim and Mansour (1972). From their original description it can be deduced that both the female and male reproductive system follows the typical platyhelminth model and display no unusual features. According to these workers the male system consists of paired testes, a cirrus pouch dorsal to the ventral sucker with seminal vesicle, pars prostatica and a coiled unspined cirrus. The female reproductive system consists of a single compressed ovary with

the Mehlis' gland immediately posterior to it. A ciliated oviduct, ovicapt (as described by Lie, 1965) are present plus a Laurer's canal opening to the dorsal surface. Additional parts of the female system consist of the ootype, uterus, uterine seminal receptacle and the extensive vitellaria. A diagrammatic representation of the female reproductive system of *E. liei* is shown in Fig 4.1.

A detailed study carried out by Lie (1965) concentrated on the Mehlis' gland complex of *Echinostoma malayanum*, *Echinostoma lindoense*, *Echinostoma audyi*, *Hypoderaeum dingeri* and a 43-spined undescribed *Echinoparyphium* species. This work was conducted on numerous live specimens. Lie observed that the structure of the Mehlis' gland complex was the same in all of the five species of echinostomes studied. Lie concluded that a small muscular structure which he termed the ovicapt, played an active role in regulating the passage of the ovum down the oviduct. His study showed that ciliated epithelium lined the ovicapt, the oviduct distal to the ovicapt and the proximal part of the Laurer's canal. The movement of the cilia was found to be directed towards the Laurer's canal from which excess sperm and vitelline granules were discharged. Spermatozoa within the female reproductive system were stored within the uterine seminal receptacle. Lie was not certain and was unable to specify where fertilisation took place, although speculating that the ootype might be the site of fertilisation. Rao (1963) suggested that fertilisation in echinostomes probably occurs most often within

Fig. 4.1 Diagrammatic representation of the female reproductive system of *E. liei*

Key

U-uterus Mg-Mehlis gland

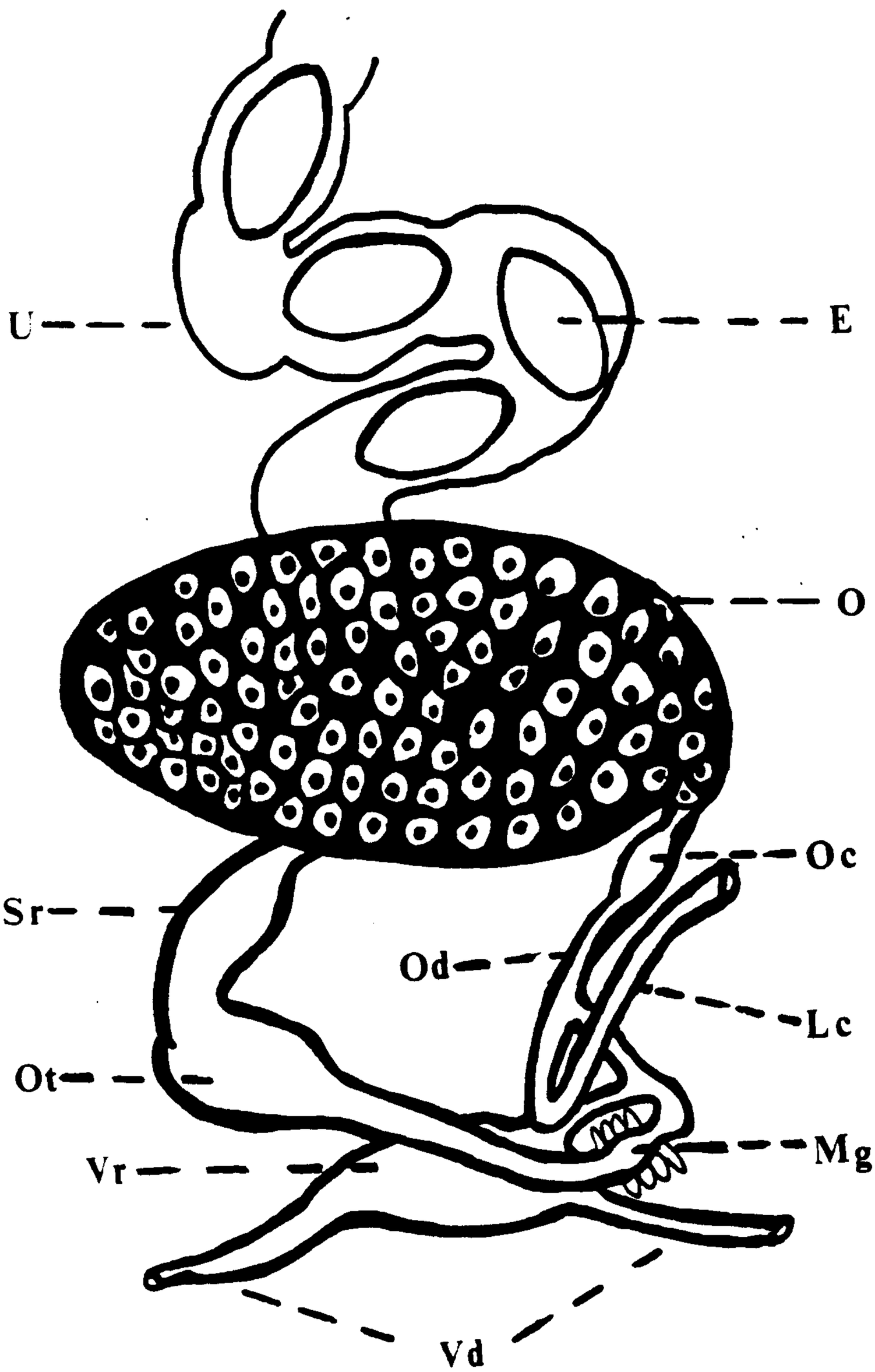
E-egg Vd-vitelline gland

O-ovary Vr-vitelline resevoir

Oc-ovicapt Ot-ootype

Od-oviduct Sr-uterine seminal receptacle

Lc-Laurer's canal


U
E
O
Oc
Sr
Od
Lc
Ot
Mg
Vr
Vd

the uterine seminal receptacle after observations on *Artyfechinostomum mehrai* recovered from human infections.

The present study was undertaken to obtain a detailed description, especially at the electron microscopical level, of the reproductive organisation of *E. liei* with specific reference to vitellogenesis and spermatogenesis. These findings will have relevance to the discussion section in Chapter 3 on vitelline gland development in maturing worms and that in Chapter 9 on deleterious alterations of spermatogenesis in worms undergoing long term selfing.

4.2 Materials and Methods

Using the routine histological light microscopical techniques outlined in Chapter 2 (Section 2.8.3.) the reproductive anatomy of sexually mature worms of *E. liei* was examined. Ten day old worms were prepared for transmission electron microscopy as described in Section 2.9.2. For investigation of spematogenesis, to make observation of the testes easier, the paired testes were dissected from the worms and prepared as outlined in Section 2.9.2.

4.3 Results

4.3.1 Overall organisation at the light microscopy level

The relationships between the positions of the male and female reproductive system can be deduced from the whole mounts in

Chapter 3 (see Fig. 3.12 (1) (2), (3), (4) and (5)), the overall female pattern (See Fig. 4.1) and the position of the vitelline glands (see Fig. 3.11). The reproductive anatomy is detailed in Fig 4.2 (1), (2) and (3). From Chapter 3 it can be seen that the uterus is intercaecal and lies between the anterior testes and ventral sucker and is filled with numerous eggs. The paired testes lie in a posterior position to the ovary and the ovary itself has a compressed appearance. The testes are arranged in tandem and occupy the posterior half of the worm body while the ovary has a relatively median position in relation to the worm body. The cirrus pouch (Fig. 4.2 (1)) contains the seminal vesicle (which contains mature spermatozoa) and a coiled conspicuous cirrus and is situated anterior to the ventral sucker without extending past its posterior margin. When the cirrus is withdrawn the position of the genital opening can be observed immediately anterior to the ventral sucker. This genital opening was explained by Chappell (1980) to be the common opening for both the male and female reproductive systems of digeneans. Fig. 4.2 (2) and Fig. 4.2 (3) show the appearance of the testes and ovary respectively in stained sections.

4.3.2 Vitellogenesis in *E. liei*

In Chapter 3 investigations with Fast Red Salt B demonstrated the presence of presumed phenolic egg shell precursors in the worms' vitelline glands from day 8 of development onwards. The follicles of the vitellaria are found in the lateral fields of the worm.

Fig 4.2 (1-3)

The reproductive anatomy of adult *E. liei*

(1) Cirrus pouch and cirrus

Scale bar= 100 μm

(2) Testes

Scale bar= 100 μm

(3) Ovary

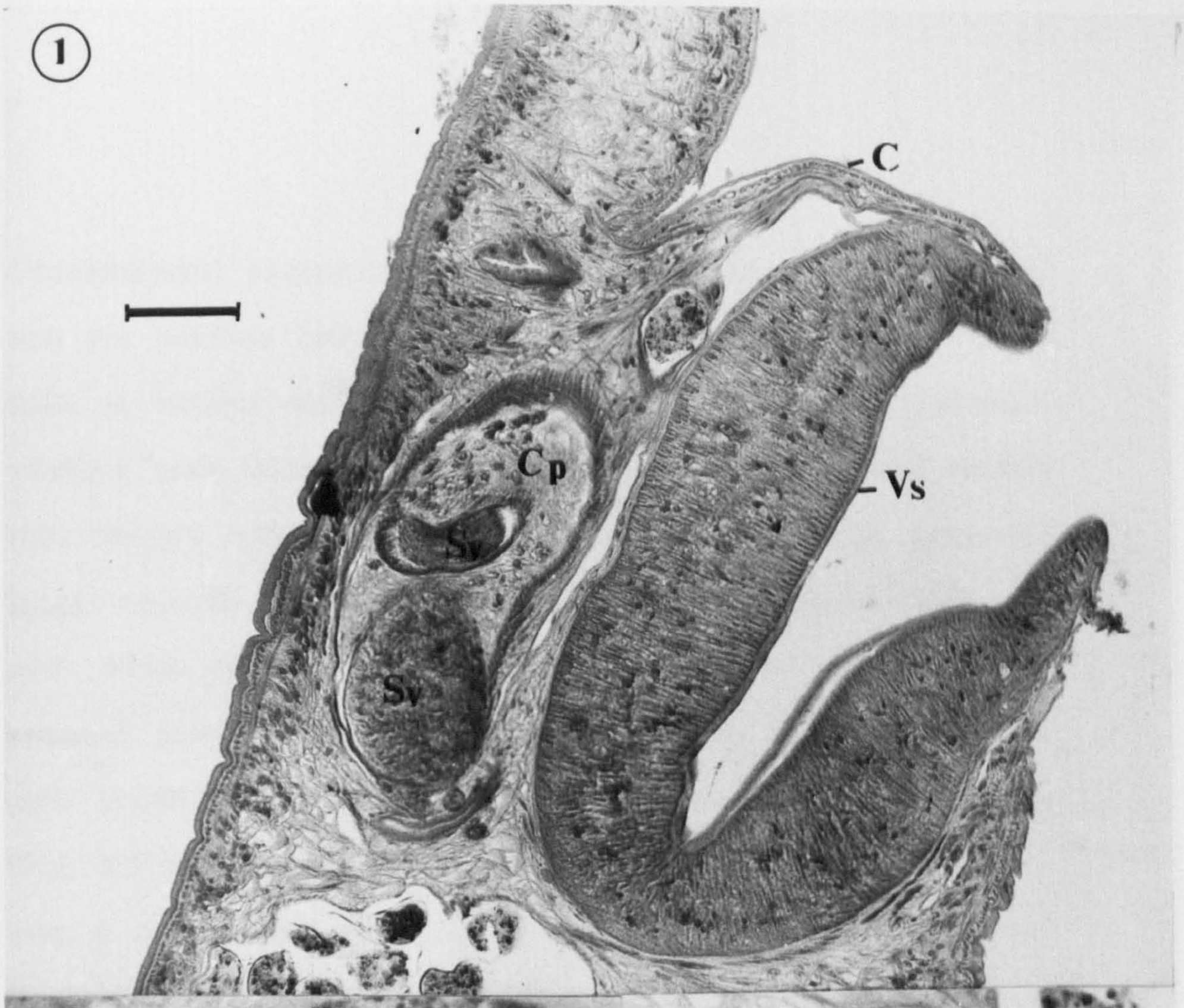
Scale bar= 100 μm

Key

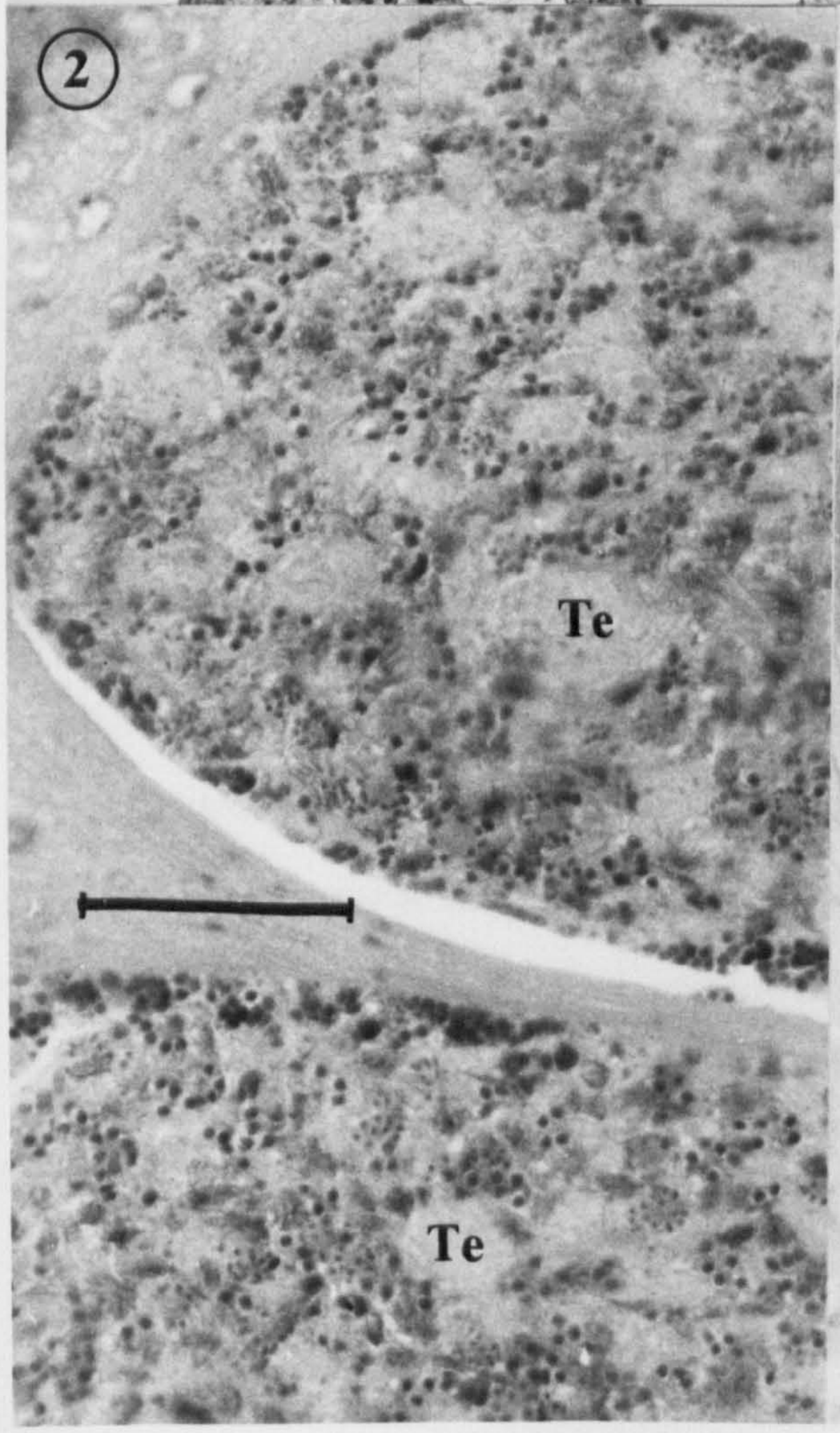
C-cirrus Cp-cirrus pouch Sv-seminal vesicle

Vs-ventral sucker Te-testes Ov-ovary E-eggs

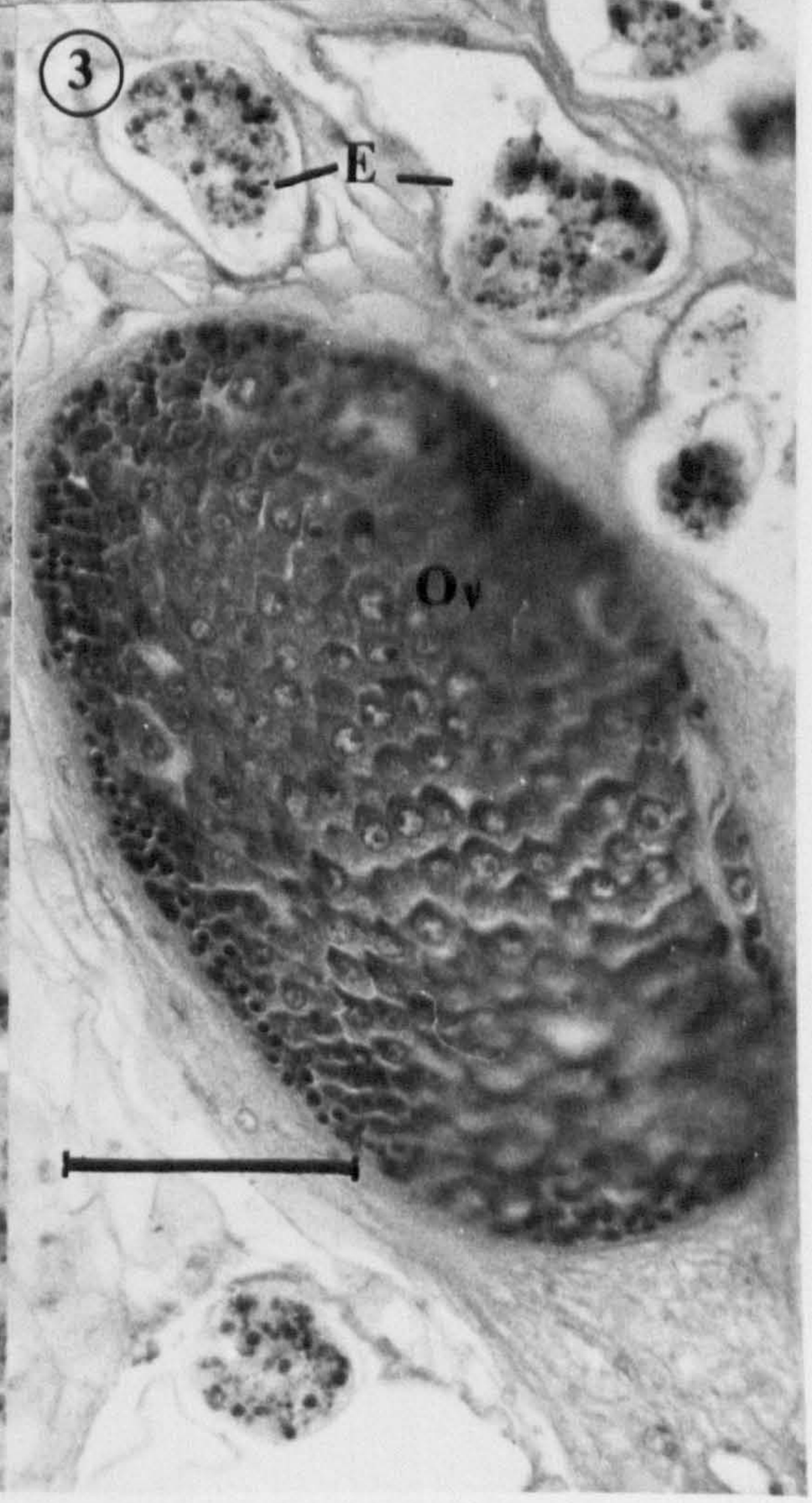
1



2



3



Ultrastructural examination of the vitellaria of *E. liei* revealed that the vitelline cells were arranged in clusters with individual cells at various stages of development. (Fig.4.3 (1)). Immature vitelline cells were characteristically undifferentiated and smaller than mature vitelline cells. Immature vitelline cells possessed large, centrally located oval nuclei (diameter approximately 3-4 μm) which occupied most of the cell volume and contained dense areas of chromatin (Fig. 4.3 (1) and (2)). The cytoplasm of these cells contained numerous small mitochondria and free ribosomes (Fig. 4.3 (2)). As these cells matured and increased in size there was a proportional increase in the area of the cytoplasm in sections, with relation to nucleus area. Further differentiation involved the formation of large quantities of granular endoplasmic reticulum (GER) in the form of parallel flattened cisternae which eventually filled much of the cytoplasm (Fig. 4.3 (3) and (4)). During the build up of GER, small electron-dense structures known as shell protein globules appear (Fig. 4.3 (3) and (4)). Associated with these globules are indistinct Golgi complexes (Fig. 4.3 (3)). The dense shell globules appear to form singly then aggregate within the cytoplasm to form approximately spherical clusters between 1-2 μm in diameter (Fig. 4.3 (4)). GER is abundant throughout the entire cytoplasm of these maturing differentiating cells. In mature vitelline cells the spherical clusters of shell protein globules congregate at the periphery of the vitelline cell (Fig. 4.3 (5)). While single isolated

Fig. 4.3 (1-6) Vitellogenesis in *E. liei*

In Fig. 4.3 (1-6) scale bar=1 μm

(1) Cluster of vitelline cells showing immature cells containing proportionately large nuclei and maturing differentiated cells containing shell protein globules at the cells' periphery.

(2) An immature vitelline cell with its large centrally located nucleus with dense areas of chromatin. The cytoplasm contains only mitochondria and free ribosome.

(3) Part of a developing vitelline cell showing extensive granular endoplasmic reticulum (GER) and the build up of shell protein globules.

(4) Clusters of shell protein globules associated with extensive GER from a maturing vitelline cell.

(5) A mature vitelline cell now containing a glycogen filled cytoplasmic region with shell protein globules accumulated at the cell periphery and a number of yolk globules in a perinuclear location.

(6) Part of a mature vitelline cell showing the edge of the nucleus and adjacent extensive glycogen deposits and yolk globules.

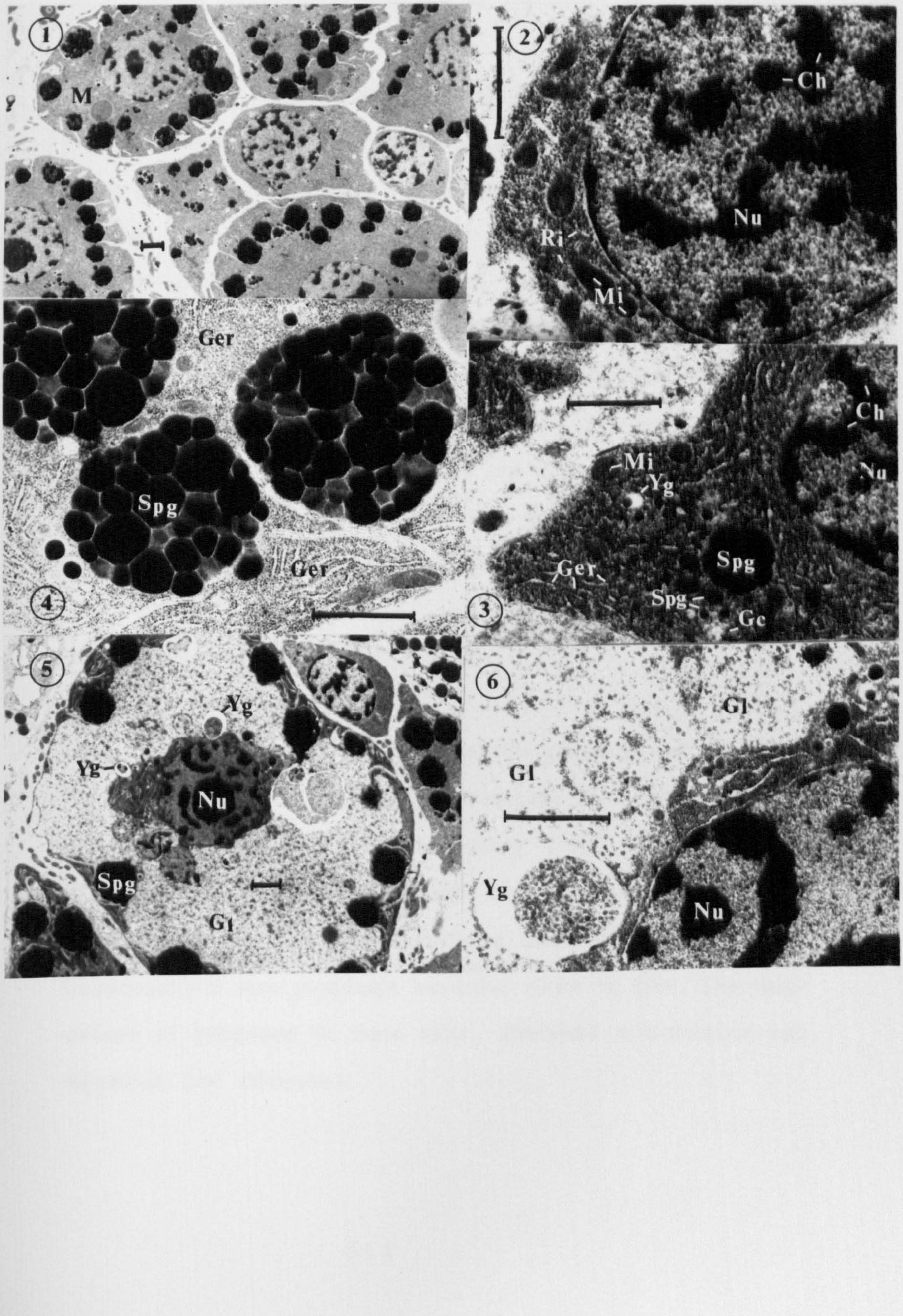
Key

i-immature vitelline cell M-maturing vitelline cell

R-ribosomes Nu-nucleus Ch-chromatin Mi-mitochondria

Spg-shell protein globules Ger-granular endoplasmic reticulum

Gc-golgi complexes Gl-glycogen Yg-yolk globules


1
M
i
I
Ger
Spg
Ger
4
2
Ch
Nu
Ri
Mi
Ch
Mi
Yg
Nu
Ger
Spg
Spg
Ge
3
5
Yg
Yg
Nu
Spg
Gl
6
Gl
Gl
Yg
Nu

shell protein globules were spherical those in clusters took on a more angular appearance, probably as the result of compression.

As the differentiating vitelline cell approaches maturity in *E. liei* , dramatic changes in the organisation of the cytoplasm take place. There is an approximately twofold increase in diameter of the vitelline cells, the nucleus becomes irregular in shape and apparent quantities of active GER are reduced (Fig. 4.3 (5)). The number of mitochondria present in the vitelline cell is reduced and much of cytoplasm becomes filled with extensive deposits of material presumed to be glycogen (Fig. 4.3 (5)). In these mature vitelline cells of *E. liei* yolk globules can also be seen surrounding the nucleus (Fig. 4.3 (5) and (6)). These yolk globules appear to be membrane-bound areas of glycogen.

4.3.3 Spermatogenesis in *E. liei*

Spermatogonia

Large spermatogonia were found near the periphery of the testes. Fig. 4.4 (1) shows a curving delineating layer separating the spermatogonia at the testis periphery from the surrounding somatic cells. These spermatogonia were characterised by large round to oval shaped nuclei containing dense areas of chromatin. Occasionally a very prominent nucleolus could be seen. The small amount of cytoplasm in these cells contained mitochondria and numerous free ribosomes.

Spermatocytes

The spermatocytes appeared to be tightly packed and contained circular nuclei with dense areas of chromatin which occured as small clumps (Fig. 4.4 (3)). The nucleus of these spermatocytes could be distinguished from that of spermatogonia in that it contains nuclear synaptonemal complexes which represent the paired homologous chromosomes of meiotic prophase (Fig. 4.4 (4)). These complexes appear as striated bands dissected by a central electron dense line. These cells contain a larger amount of cytoplasm when compared with the spermatogonia and also evident within their cytoplasm were numerous unattached ribosomes and mitochondria.

Spermatids

Early and late spermatids at various stages of development with condensing nuclei were observed in *E. liei* in both transverse (Fig. 4.4 (5), (6) and (8)) and longitudinal (Fig. 4.4 (7)) positions. The nuclear chromatin seemed to fill the entire nucleus and appeared to be distributed in clumps or as short dense filaments, very different from the arrangement of the chromatin of both the spermatogonia and the spermatocytes. The nucleoli appeared to be small and compact and in some instances were very prominent (Fig. 4.4 (5)). These cells also had a greater amount of cytoplasm when compared to both the spermatogonia and spermatocytes and contained numerous free ribosomes. These spermatids were characterised by mitochondria which occupied a perinuclear

Fig. 4.4 (1-8) Spermatogenesis in *E. liei*

(1) Spermatogonia located near the periphery of the testes

Scale bar= 1 μm

(2) Cross section of two basal bodies from a spermatid. Note the 9x3 microtubular organisation of the centrioles and the peripheral microtubules.

Scale bar= 0.1 μm

(3) Spermatocytes

Scale bar= 1 μm

(4) Nuclear synaptonemal complex from spermatocyte nucleus

Scale bar= 0.1 μm

(5) Spermatid nucleus surrounded by fused mitochondria

Scale bar= 1 μm

(6) Spermatid nucleus encircled by fused mitochondria

Scale bar= 1 μm

(7) Elongate spermatid nucleus with mitochondria in a perinuclear position

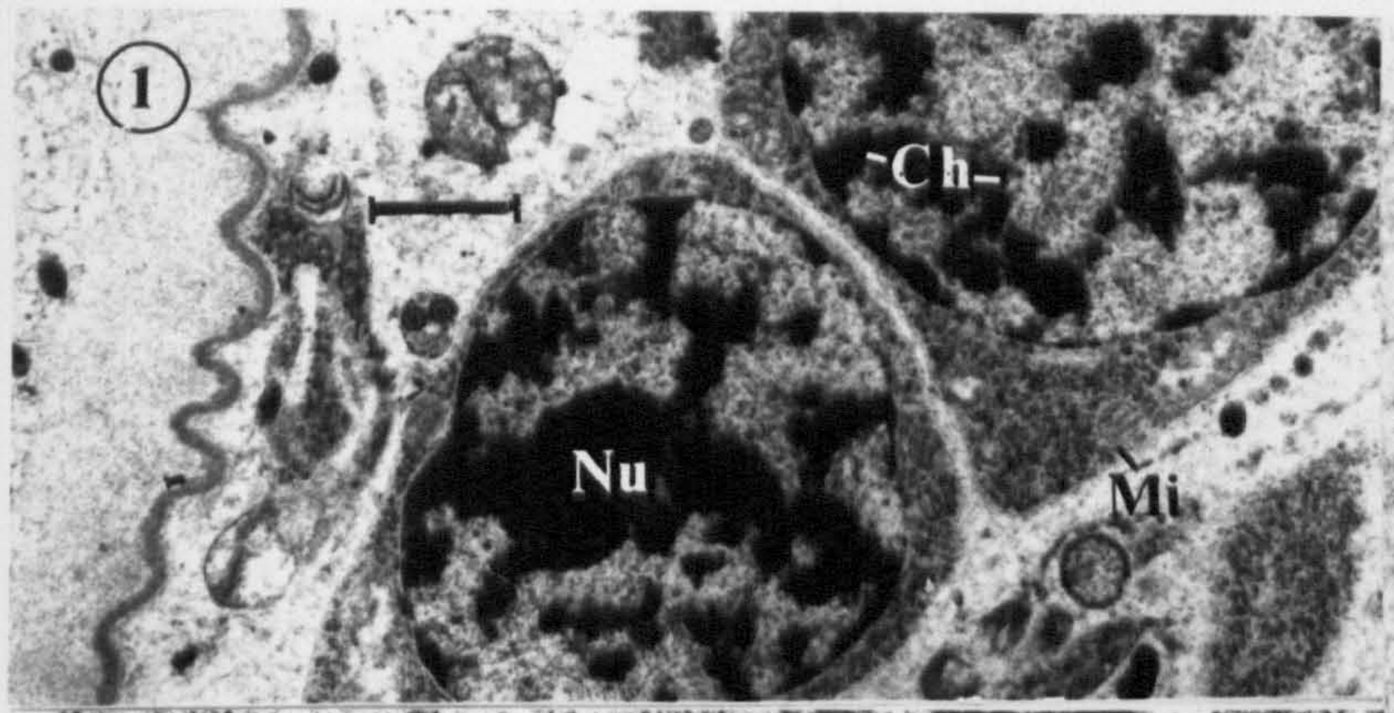
Scale bar= 1 μm

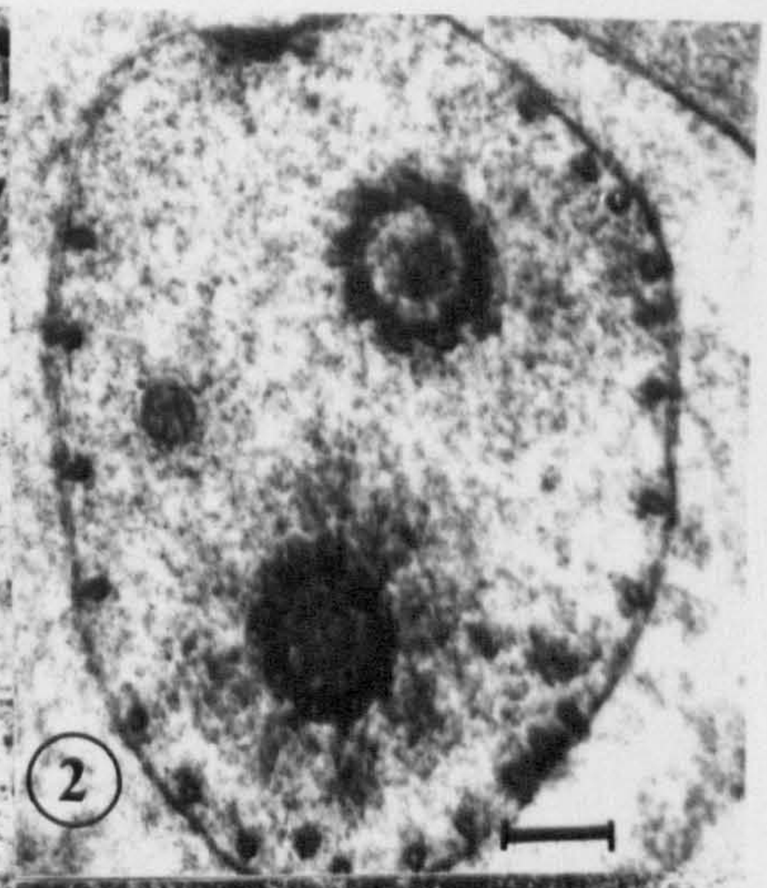
(8) Spermatid nucleus containing chromatin in the form of scroll-like lamellae

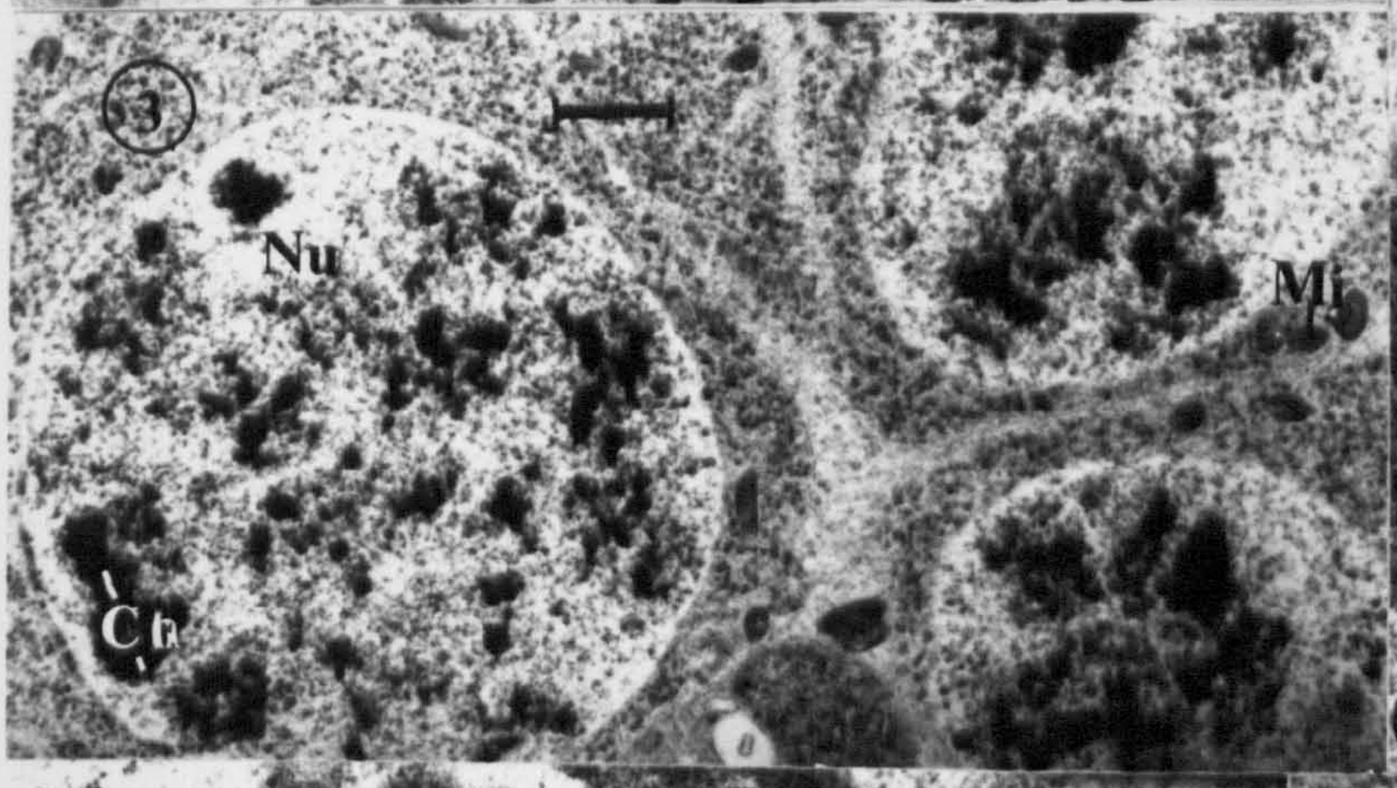
Scale bar= 0.5 μm

Key

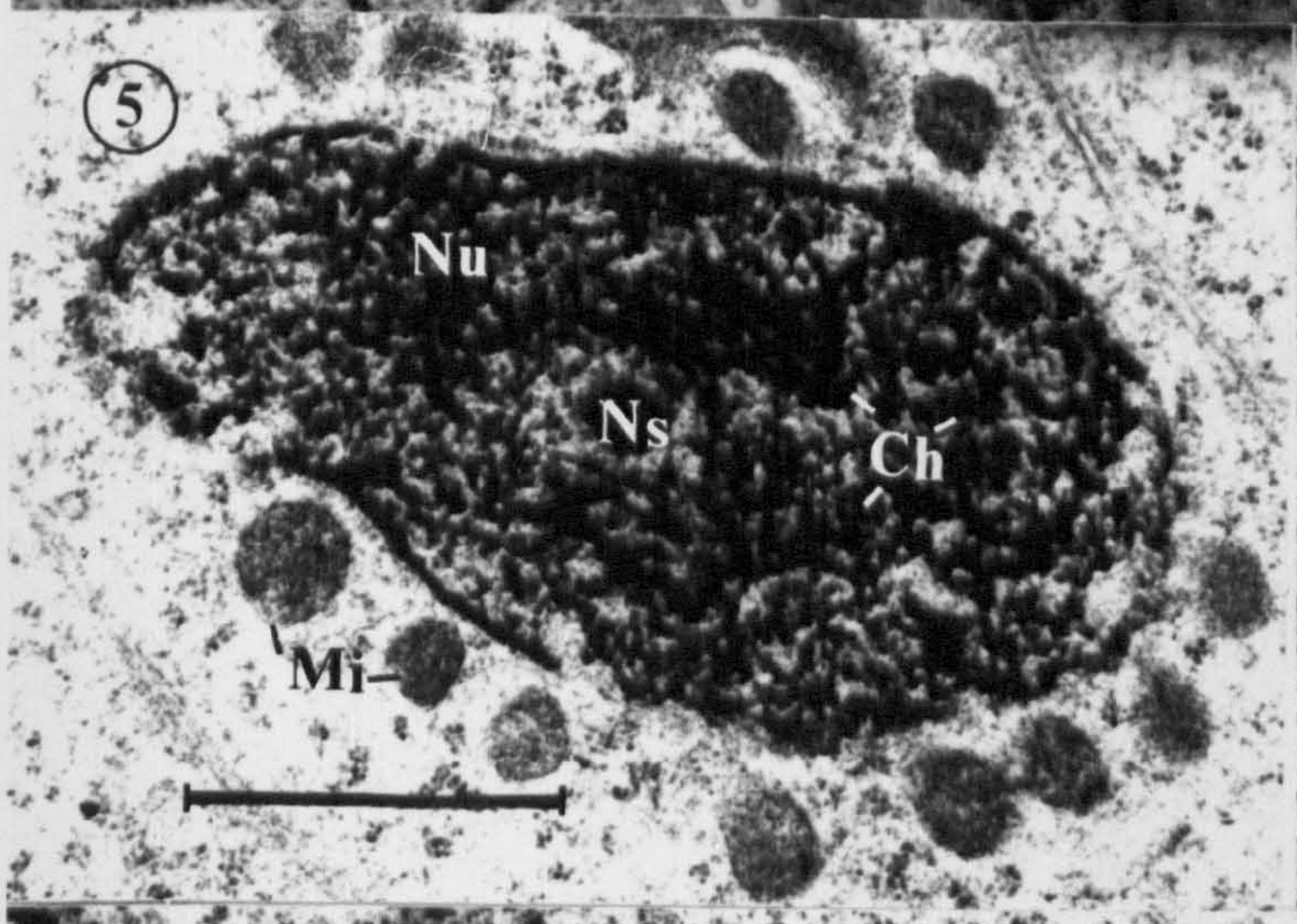
Nu-nucleus Ch-chromatin Mi-mitochondria Ns-nucleoli

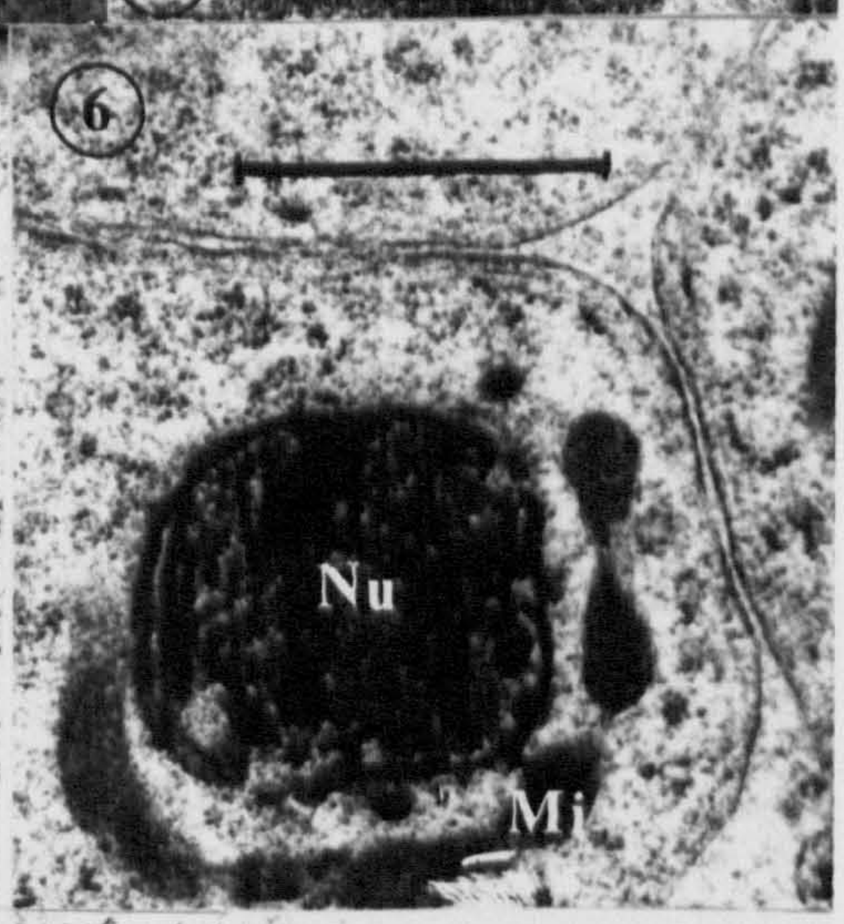

1
Ch
Nu
Mi

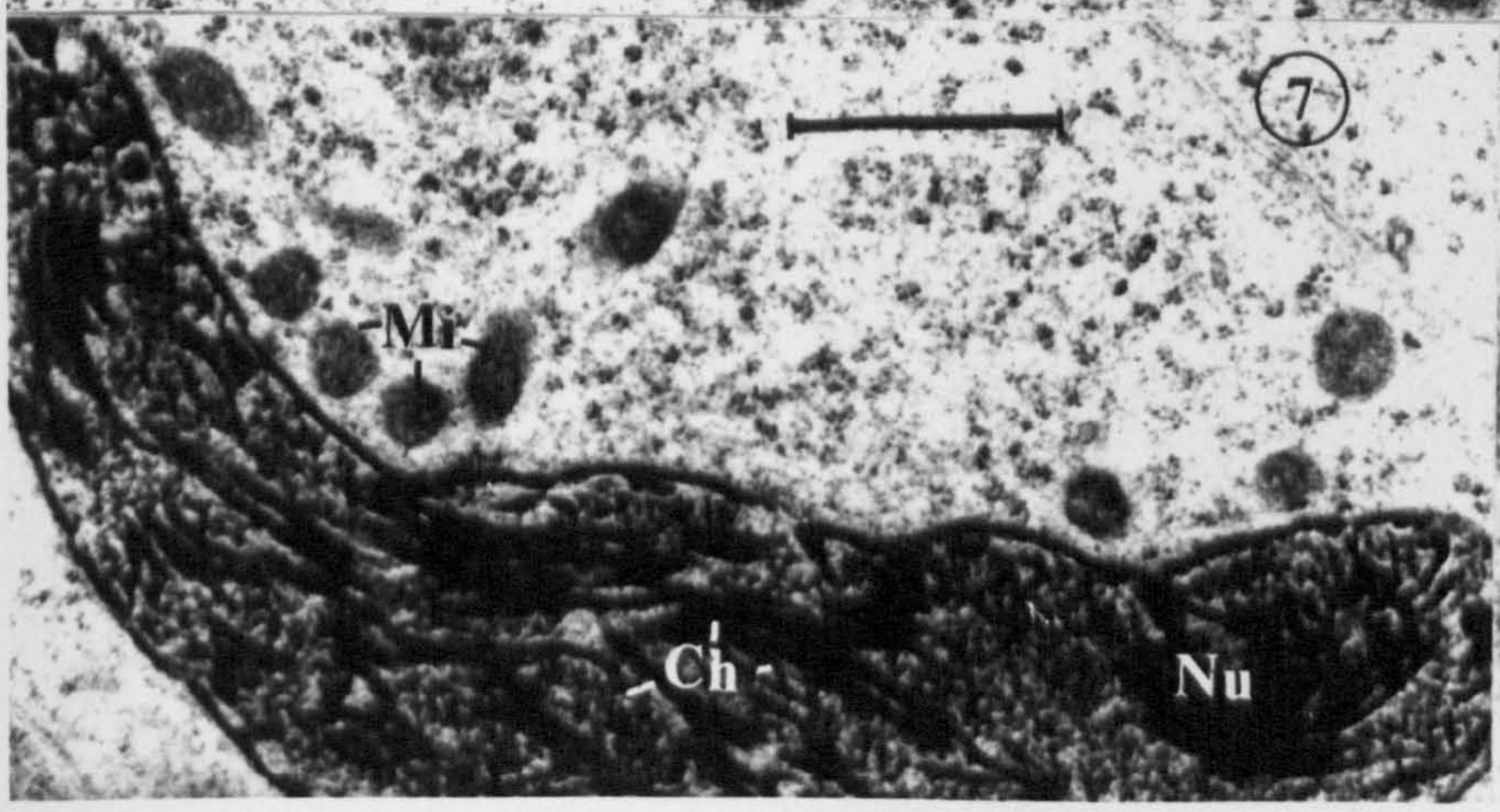

2


3
Nu
Mi
Ch


4


5
Nu
Ns
Ch
Mi


6
Nu
Mi


7
Mi
Ch
Nu

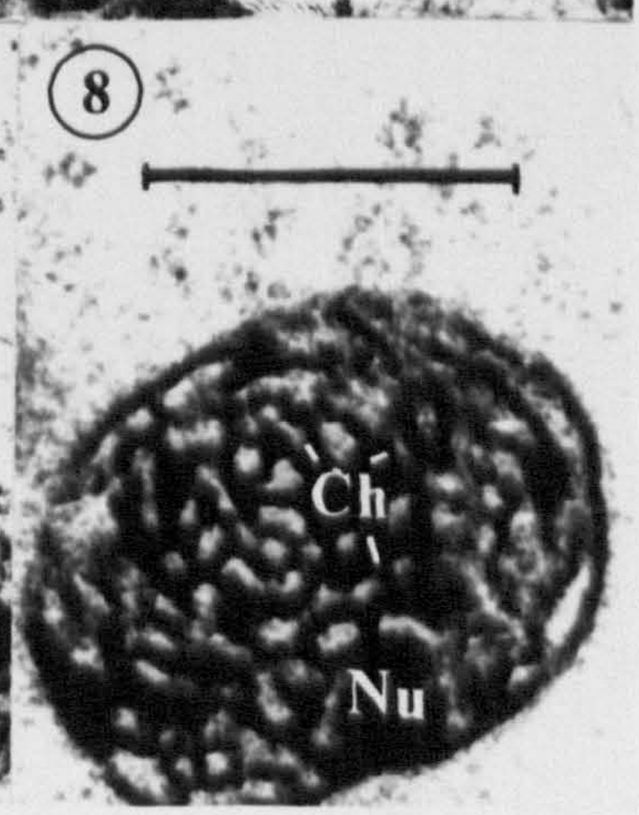

8
Ch
Nu

Fig. 4.5 (1-12) Spermatogenesis in *E. liei*

(1) Section through a differentiating spermatid in *E. liei* at the zone of differentiation

Scale bar= 1 μm

(2) The zone of differentiation, axial filaments, rootlets and central body of a differentiating spermatid.

Scale bar= 1 μm

(3) Section through the basal bodies of the paired axial filaments showing the central body orientated at right angles to the basal bodies

Scale bar= 0.5 μm

(4) Transverse section through a region of the median process and the axial filaments

Scale bar= 0.5 μm

(5) Transverse sections of unattached axial filaments

Scale bar= 0.1 μm

(6) Axial filament showing the 9+1 arrangement of the microtubules

Scale bar= 0.1 μm

(7) Transverse sections through developing spermatozoa

Scale bar= 0.5 μm

(8) as (7) Scale bar= 0.2 μm

(9) Terminal region of spermatozoon

Scale bar= 0.1 μm

(10) Terminal region

Scale bar= 0.1 μm

(11) Section through the terminal region of a structurally abnormal spermatozoon, showing a double pair of axial filaments

Scale bar= 0.2 μm

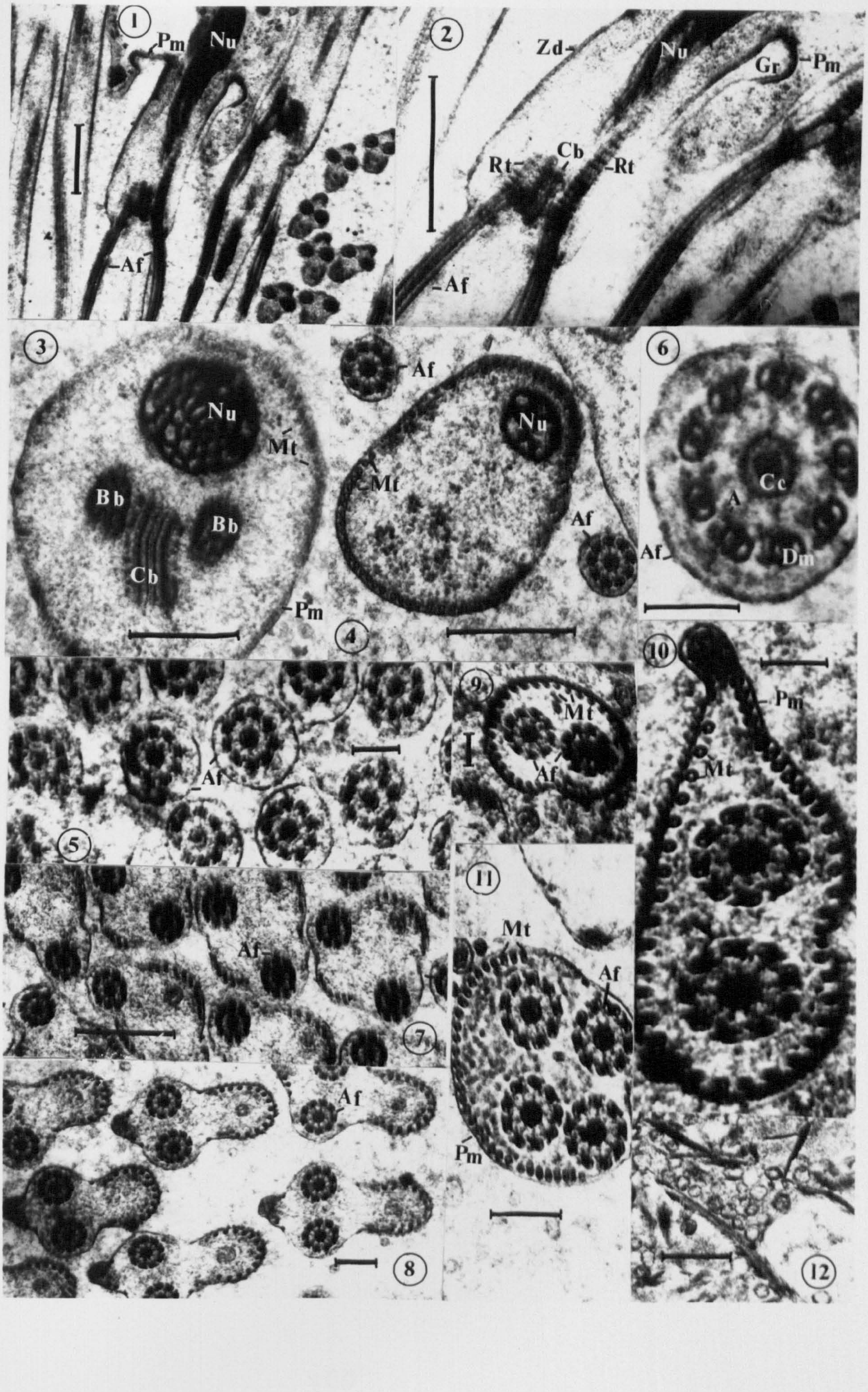

1
Pm
Nu
Af
2
Zd
Nu
Gr
Pm
Rt
Cb
Rt
Af
3
Nu
Mt
Bb
Bb
Cb
Pm
4
Af
Nu
Mt
Af
6
Cc
A
Af
Dm
5
Af
7
Af
8
9
Mt
Af
10
Pm
Mt
11
Mt
Af
Pm
12

Fig. 4.5 (Cont.)

(12) Spermatozoan autolysis

Scale bar= 0.5 μm

Key

Nu-nucleus Af-axial filament Pm-plasma membrane

Zd-zone of differentiation Cb-central body Gr-groove

Rt-rootlets Bb-basal bodies Mt-microtubules

Dm-doublet microtubules

A-arms of doublet microtubules Cc-central complex

position and formed a circle around the nuclei of these cells in section (Fig. 4.4 (5) and (7)). In later stages, these mitochondria having encircled the nuclei of the spermatid appeared to fuse (Fig. 4.4 (6)) giving the appearance of an elongate mitochondria surrounding the nucleus. Other spermatids presumed to be at a more advanced stage, contained chromatin which had condensed into a reticulum of dense scroll-like lamellae and filled the entire nucleus (Fig. 4.4 (8)). The nucleus of these cells appeared initially oval-shaped but later elongated as the condensation of the chromatin took place. The condensation of the nuclear chromatin coupled with the enlargement and elongation of the nucleus and the re-alignment of the mitochondria marks the onset of spermiogenesis.

Concomitant with this nuclear activity incorporating both condensation and elongation in *E. liei,* is the formation at the distal end of the cell, of lateral extensions. This region is known as the zone of differentiation and the extensions mark the formation of two lateral axial filaments (Fig. 4.5 (1) and (2)) each being delimited by the plasma membrane. This zone of differentiation is clearly set apart from the spermatid at its basal end where the plasma membrane appears to curl back on itself forming a groove which marks the base of the zone of differentiation. The nucleus of the spermatid clearly extends into this zone of differentiation (Fig. 4.5 (2)). In it the basal bodies and axial filaments occur at right angles to the central body which

has a fibrous striated appearance (Fig. 4.5 (3)). The two basal bodies (Fig. 4.4 (2)) serve as nucleation sites for the polymerisation of the doublet tubules of the developing axial filaments. The central body appears as a distinctive array of parallel electron dense bodies and has been commented on by numerous workers. A similar structure has been referred to as a centriole by Silveira and Porter (1964), a centriole-like body (Burton, 1972), a microtubule organising centre (Grant, Harkema and Muse 1976) and a centre body (Erwin and Halton 1983). In this study the terminology used by Rees (1979) and Hendow and James (1988) is adopted.

Early in spermiogenesis striated rootlets polymerise in association with basal bodies (Fig. 4.5 (2)). The developing lateral projecting axial filaments appear to be anchored by these long fibrous rootlets which emanate from each basal body and appear as a number of repeated dense transverse striations. These rootlets come to extend deep into the cytoplasm of the spermatid one extending further than the other. During these events the two axial filaments have extended laterally from the zone of differentiation. The nucleus extends into the developing median cytoplasmic process, which develops as an extension of the zone of differentiation distal to the central body (Fig. 4.5 (4)). Also associated with the plasma membrane in the vicinity of the median process are a number of peripheral microtubules present

along the inside of the plasma membrane at both the proximal and distal end.

It appears that there is a cytoplasmic continuity of adjacent cells at the site of membrane invagination at this stage of differentiation of the developing spermatids. The cytoplasm in this kind of syncytial arrangement during helminth spermatogenesis has been termed the cytophore (McLaren, 1973) and has been found to contain numerous ribosomes. During spermiogenesis, then, the nucleus of the spermatid appears to become reduced in volume and passes into the cytoplasmic median process of the developing spermatozoon. The central cytoplasmic median process and the two axial filaments ultimately fuse forming a unipartite structure (Burton, 1972) which is characteristic of the digenean spermatozoon (Rees, 1979).

Spermatozoa

Ultrastructural examination of the testes revealed a large number of immature spermatozoa present in the testes at different stages of development. At different sections of the elongate developing spermatozoa the two axial filaments (each with a characteristic 9+1 microtubular organisation) may either be embedded in the spermatozoan cytoplasm or extend freely surrounded by their own flagellar plasma membrane. Figs. 4.5 (7), (8), (9) and (10) show profiles of spermatozoa in the former condition (that is,

axial filaments within the spermatozoan body). Figs 4.5 (5) and (6) illustrate free flagella profiles. In the latter, details of the microtubular organisation of the axial filament are demonstrated in Fig. 4.5 (6). Each axial filament consists of nine doublet microtubules arranged in a cylinder around a central complex (Fig. 4.5 (6)). This arrangement of the tubules of the axial filament has come to be known as the 9+1 arrangement (Shapiro, Hershernov and Tulloch (1961). Henley, Costello, Thomas and Newton (1969) correctly pointed out that the 9+1 designation for the microtubule pattern is not an accurate one since the central core is not composed of one singlet microtubule as the term would imply but has a complex structure. Nine radial spoke-like strands from the doublet microtubules are connected to the central complex. There also appear to be discrete connections between the microtubule doublets and the axial filament membrane. The two microtubules of each doublet are morphologically distinct in that one set of each doublet bears a pair of arm-like processes which all point in the same direction. The central complex consists of a central electron dense-core fibril separated from an outer dark ring or central sheath of similar density by a central matrix which is electron-lucent.

Peripheral microtubles are prominent in many profiles (Figs 4.5 (7), (8), (9) and (10)) sometimes in a single unilateral band (Fig. 4.5 (8)) on other occasions in two opposite bands (Fig. 4.5 (7)) while in yet others the microtubules appear to extend around the

whole periphery (Figs. 4.5 (9) and (10)). It can be seen clearly in these transverse sections of developing stages of spermatozoa that the cortical tubules run along the whole length, attached to the inner surface of the plasma membrane. Sections of spermatozoa in Figs 4.5 (7) and (8) both contain spherical structures which are probably sections through the mitochondrion. It has proved difficult to produce an unambiguous interpretation of the extent to which the condensed nucleus and fused mitochondrion extend down the elongate spermatozoon.

On very rare occasions aberrant spermatozoa containing a double pair of axial filaments were seen (Fig. 4.5 (11)). Observations of the testes of *E. liei* also revealed distinct areas which appeared to show mass tissue disintegration indicating an autolytic component of the testes (Fig. 4.5 (12). This component of the testes may deal with the intracellular degradation of unwanted testicular material.

4.4 Discussion

The overall organisation of the male and female reproductive system of *E. liei* demonstrates no unusual features and exhibits the basic pattern of hermaphroditic digeneans (Smyth and Halton, 1983; Chappell, 1980). The prominent features of the male system being the paired testes, seminal vesicle and cirrus. Within the female system these features are a single ovary, a uterus and vitellaria.

At the ultrastructural level the vitelline cell clusters of *E. liei* have cells in progressive stages of maturation comparable with those described for other platyhelminths. There are no other comparative vitellogenesis studies at the electron microscopical level of the 37-spined echinostomes. The structure of the cells and the sequence of events bear a fundamental similarity to vitelline cell development observed in the digeneans, *Haemtoloechus medioplexus* (Tulloch and Shapiro, 1957) *Fasciola hepatica* (Irwin and Threadgold, 1970) *Schistosoma mansoni* (Shaw, 1987), the monogeneans *Diplozoon paradoxum*, *Diclidophora merlangi*, *Diclidophora denticulata*, *Calicotyle kroyeri* (Halton, Stranock and Hardcastle, 1974) and the cestode *Trilocularis acanthiaevulgaris* (Mahendrasingam, Fairweather and Halton, 1989). Also the sequential vitelline cell development resembles that of the triclad *Dugesia lugubris* (Domenici and Gremigni, 1974; Gremigni and Domenici, 1974). Threadgold's (1982) stereological analysis of vitelline cell development in *F. hepatica* identified four stages of vitelline cell ontogeny and showed that during maturation there was a significant increase in nuclear size, an increase in the number of mitochondria which correlated with the energy demands of growth and synthesis and a 16-fold increase in the total volume of GER, coupled with an increase in the total cytoplasmic volume. In both *E. liei* and the digenean *Maritrema linguilla* (Hendow and James, 1989) vitellogenesis occurs as the cytoplasm increases in volume.

Hendow and James (1989) referred to vitellogenesis in *F. hepatica* and *S. mansoni* as primitive when compared to that of *M. linguilla* and pointed out that entire whole vitelline cells are incorporated into the eggs of these primitive forms as well as the utilization of the shell protein clusters and the food reserves.

Smyth and Clegg (1959) clearly established that the shell globules are primarily protein while Mahendrasingam *et al.* (1989) demonstrated positive results for phenolase, phenols and basic proteins in the shell globules of the cestode *T. acanthiaevulgaris*. Irwin and Threadgold (1970) hypothesised that this protein is synthesised in the extensive GER and transferred to the Golgi complex where it is concentrated, eventually producing a membrane bound spherical globule. These single globules subsequently fuse forming large shell globule clusters that aggregate at the vitelline cell periphery. Halton, Stranock and Hardcastle (1974) have also suggested that Golgi complexes are the organelles that give rise to the numerous dense shell globules which characterise the maturing cell. It is highly probable that the phenolic material identified in *E. liei* (See Chapter 3) is located in these shell globule clusters since studies have shown that the phenolic protein and phenolase, the enzyme which brings about tanning, are both located in the shell globule clusters (Smyth and Clegg, 1959). Similar observations were made by Gremigni and Domenici (1974) while studying the vitelline glands of the freshwater triclad *Dugesia lugubris*. They explained

that the components of the cocoon-shell involved the synthesis of proteins and non-phenolic proteins which occurred in the cisternae of the endoplasmic reticulum.

Irwin and Threadgold (1970) explained that mature vitelline cells were filled with glycogen rosettes. Structures referred to as yolk globules by Bjorkman and Thorsell (1963) were present within the cytoplasm and occupied a perinuclear position. Irwin and Threadgold (1970) described the yolk globules found in *F. hepatica* as being membrane bound areas of concentric GER and glycogen. Similar structures were observed in the vitelline cells of the fish-gill monogeneans *Diplozoon paradoxum*, *Diclodophora merlangi*, *Diclidophora denticulata* and the fish-skin monogenean *Calicotyle kroyeri* (Halton, Stranock and Hardcastle, 1974).

Orido (1988) has shown that in *Paragonimus ohirai*, enclosed by the egg shell a single oocyte coalesces with 9-10 vitelline cells located at the opercular end. Orido observed during oogenesis that round dense granules are released from vitelline cells and deposited to form the wall of the eggshell around the oocyte and the rest of the vitelline cells. In *F. hepatica* (Irwin and Threadgold, 1970) and in *D. paradoxum*, *D. merlangi*, *D. denticulata*, *C. kroyeri* (Halton, Stranock and Hardcastle, 1974) it has been shown that the mature vitelline cell is released into the vitelline duct.

Irwin and Threadgold (1970), Halton Stranock and Hardcastle (1974) and Smyth and Halton (1983) have all commented upon the dual role of the vitelline cell during development. That is, that it initially functions as a protein synthesising cell (providing the precursors for the egg shell) and then as a food storage unit for the development of the fertilised egg. In *E. liei* the synthesised protein in the form of single, spherical membrane bound dense globules, migrates to the cell periphery where large clusters are formed and accumulate. The origin of the glycogen of the vitelline cells is not known but its appearance coincides with the disappearance of the GER. Smyth and Clegg (1959) have postulated that the glycogen of the vitelline cells is utilized as food reserves by developing miracidia. The vitelline cells are therefore clearly multi-functional with glycogen and yolk globules available as food reserves for the developing embryo, and shell protein clusters providing the structural proteins for egg shell formation (Smyth and Halton, 1983).

Studies and on aspects of spermatogenesis in the Digenea at the ultrastructural level have included reports on *Pharyngostomoides procyonis* (Grant, Harkema and Muse, 1976), *Cryptocotyle lingua* (Rees 1979), *Corrigia vitta* (Robinson and Halton, 1982) *Bucephaloides gracilescens* (Erwin and Halton, 1983) and *Maritrema linguilla* (Hendow and James, 1988). Within the genus *Echinostoma* there is a lack of descriptive insight at the ultrastructural level into the sequence of spermatogenetic events.

The complexity of the process is evident in all of the studies carried out in digeneans but the typical ultrastructural sequence has been outlined by Burton (1972) in his classic study on the spermatozoon and its differentiation in the lung fluke *Haematoloechus medioplexus*. Burton explained that a single spermatogonial cell ultimately gives rise to a rosette of 32 spermatids. The spermatids then undergo a complex process of differentiation to produce spermatozoa. This pattern of development was also described by Gresson (1965) in his review of spermatogenesis in the hermaphroditic Digenea. Orido and Hata (1988) stated, as a result of cytological observation of spermatogenesis in *Paragonimus ohirai*, that mitotic divisions of a primary spermatogonium produced the eight cell stage of the primary spermatocytes, which subsequently meiotically produced secondary spermatocytes at the sixteen cell stage which by further meiotic divisions produced spermatids at a 32 cell stage. Robinson and Halton (1982) and Hendow and James (1988) after observing spermatogonia in *C. vitta* and *M. linguilla* respectively, commented that spermatogonia divide by mitosis to give first secondary and then tertiary cells which in turn divide to give 8 primary spermatocytes. In this study it has not been possible to recognise morphological or ultrstructural features which would enable putative primary, secondary or tertiary spermatogonium to be distinguished.

According to Gresson (1965), Robinson and Halton (1982) and Erwin and Halton (1983) the 8 primary spermatocytes undergo synchronous meiotic divisions to form 16 secondary spermatocytes which produce meiotically a rosette of 32 spermatids. Interestingly, Hendow and James (1988) noted that in *M. linguilla* spermiogenesis began with the appearance of up to 75 cross-sections of microtubules on the apical margin of the spermatid which they explained would elongate to form the zone of differentiation.

The precise origin and function of the central body identified in *E. liei* is not known although Rees (1979) has speculated that it may possibly be associated with the central complex of the axial filament which has no template in the basal body. Robinson and Halton (1982) believed that it may participate in the construction of cortical microtubules which extend into the median process.

In *E. liei* the rootlets at the zone of differentiation extend deep into the cytoplasm of the spermatid, one extending further than the other a feature comparable to the assymetry of the rootlets arranged on either side of the central body as reported by Rees (1979) in *C. lingua.* In contrast Erwin and Halton (1983) observed that the rootlets in *B. gracilescens* exhibited a symmetry arround the central body. The peripheral microtubules associated with the median process (Fig. 4.5 (4)) have been suggested of having a cytoskeletal role in stabilising and supporting the zone of

differentiation (Erwin and Halton, 1983; Burton, 1972). Along the inside of the developing spermatozoon plasma membrane, these microtubules have been suggested to be involved in motility (Thomas and Henley, 1971) and it appears in *E. liei* that they run the whole length of the spermatozoon.

The 9+1 arrangement of the microtubules in the axial filament was first reported by Shapiro, Hershenov and Tulloch (1961) and is a characteristic feature of many platyhelminth spermatozoan flagella. Kessel (1966), Burton (1972) and Jamieson and Daddow (1982) have proposed a cytoskeletal locomotory or transport function for these microtubules of the axial filaments. It has been speculated that the cytoplasm between and around the incorporated axial filaments might play a role in both rigidity and resistance of bending of the mature sperm (Henley, Costello Thomas and Newton, 1969). Burton and Silveira (1971) have commented that the radial spokes and central complex in playthelminth sperm may function to maintain the spatial or cylindrical integrity of the sperm.

Robinson and Halton (1982) observed abnormal mature spermatozoa with multiples of pairs of axial filaments in the seminal receptacle of *C. vitta* and explained that such irregular conformations were a common occurrence. Aberrant multiflagellated spermatozoa commonly occur among normal uniflagellated sperm in the testis of vertebrates (Phillips, 1974).

Phillips explained that multiple flagella usually occur in groups of 2, 4 or 8 and they may arise as a direct result of 1, 2 or 3 abnormal cycles of centriole formation.

In the report carried out by Rees (1979) on spermiogenesis in *C. lingua* it was noted that the 32 spermatozoa of a cluster are eventually liberated into the testes lumen leaving a residual mass of cytoplasm, that is, the original cytophore where cellular disintegration takes place. These areas may correspond to the apparent autolytic regions observed in *E. liei*. Robinson and Halton (1982) have pointed out that this residual mass contains unattached ribosomes degenerating mitochondria Golgi saccules profiles of endoplasmic reticulum and myelin bodies. The fact that basal bodies, rootlets and the central body were never observed in the liberated spermatozoon (Burton, 1972) led Rees (1979) to speculate that these structures remain in the residual cytoplasm and presumably degenerate.

It is evident from this study that in *E. liei* the axial filaments have become incorporated into a unipartite spermatozoon, a characteristic feature of sperm of the Digenea (Jamieson and Daddow, 1982). Separation of the spermatozoon has been found by Robinson and Halton (1982) to occur at the apex of the zone of differentiation. In *E. liei* the abundant occurrence of free flagella and the less frequent fused tails may indicate that mature spermatozoa are only rarely present in the testes. It would thus

appear that the distal migration of the nucleus is very rapid and the spermatozoa leave the testes as soon as they are formed.

Up to the point in spermiogenesis in *E. liei* studied in this investigation, the processes of spermatogenesis and spermiogenesis in this species seem very similar to those described for other digeneans such as *B. gracilescens* (Erwin and Halton, 1983), *C. vitta* (Robinson and Halton, 1982) and *M. linguilla* (Hendow and James, 1988). Although fully mature spermatozoa have not been described in this study, the late stage spermatids which have been investigated suggest that the mature male gamete of *E. liei* will be very similar to other previously described digenean spermatozoa such as those decsribed by Jamieson and Daddow (1982) in *Neochasmus* sp. Orida and Hata (1988) commented on the fact that both spermatogenesis and oogenesis in digeneans are processes that are characteristic among genera even species.

With respect to the cellular material seen within the spermatozoa, Robinson and Halton (1982) used specific staining to indicate that spermatozoa of *C. vitta* contain moderate amounts of glycogen which at the ultrastructural level appeared dispersed throughout the cytoplasm and the axial filaments. These they pointed out acted as potential energy stores for active movement over short distances. Erwin and Halton (1983) pointed out that these stores were common in spermatozoa of many digeneans but in *B.*

gracilescens found that glycogen was only present in the vas deferens and seminal vessicle. These workers found that the ultrastructure of the walls of the vas deferens and the seminal vessicle were consistent with that of secretory tissue implicating that these structures may serve to supply the migrating spermatozoa with substrates for glycogen synthesis.

At the ultrastructural level fertilisation in digeneans is seen to be attained by the fusion of the plasma membrane of a single spermatozoon with a mature oocyte (Burton, 1967; Orida and Hata, 1988). This has been shown to be followed by the passage of the spermatozoon nucleus, mitochondria and axial filaments into the oocyte and the association of the spermatozoon microtubules with the oocyte surface (Orida and Hata, 1988).

CHAPTER 5

MICROHABITAT UTILIZATION BY *ECHINOSTOMA LIEI* IN THE MOUSE HOST DURING THE COURSE OF INFECTIONS

5.1 Introduction

Studies of a wide range of parasitic associations have revealed that specimens of individual species of parasites are not spatially distributed at random throughout the bodies of their hosts but utilize and occupy species-characteristic locations (Whitfield, 1979). Many parasites also exhibit complex and subtle migratory behavioural patterns within their hosts (Ulmer, 1971; Crompton, 1973; Holmes, 1973) and these often appear to move parasites to locations which maximally fulfill their particular physiological requirements thus enabling a parasite to occupy a suitable microhabitat (Read, 1971). On entry of an endoparasite into its definitive host, migratory activity removes the parasite from its site of entry to a final and favourable position (Chappell, 1979). These migrations, particularly in the cases of endoparasitic helminths, are often accompanied by growth and development which result in the mature adult worm ultimately residing in its optimum location in the host, one where it is assumed it can exhibit maximal reproductive success.

Parasitic migrations of this type have been shown to be a characteristic attribute of many different gut-inhabiting helminths in their definitive hosts (Crompton, 1973). Turton (1971), for instance, studied the distribution and growth of *Hymenolepis diminuta* in the rat, hamster and mouse. He observed anterior migrations of *H. diminuta* in both rats and hamsters over three weeks but did not observe any migrations in mice. Radlett (1979) observed the excystation of the digenean *Notocotylus attenuatus* in the domestic fowl and its posterior migration from the site of excystation, the lower intestine, into the caeca.

Ontogenetic migrations of this type are characterised by parasitic migratory behaviour which is specifically associated with parasite maturation in the definitive host. The ontogenetic migrations of adult echinostome worms in their definitive hosts have received some attention, but most work has focused on the identification of the preferred site for growth and reproduction of the adult worms with some emphasis on developmentally related migration (Hsu, Lie and Basch, 1968; Jeyarasasingam, Heyneman Lim and Mansour, 1972; Fried, 1984; Fried and Freeborne, 1984; Franco, Huffman and Fried, 1988).

Hsu, Lie and Basch (1968) recorded the presence of *Echinostoma rodriguesi* in the intestine of experimentally infected hamsters, chicks, pigeons and mice. Jeyarasasingam *et al.* observed *E. liei* in the mid-small intestine in hamsters and the small intestine of chicks. Fried (1984) recovered worms of *E. revolutum* in chicks from the ileum, caeca, rectum-cloaca and the bursa of Fabricius but did not comment on the possible reasons for this broad spatial distribution. Fried and Freeborne (1984), studying the effects of worm crowding in *E. revolutum* infections in the chicken alimentary tract, recovered worms from the ileum, cloaca, rectum, bursa of Fabricius and caeca. These workers noted significant *E. revolutum* aggregation in chicks infected with 100 metacercarial cysts. They observed that *E. revolutum* in such infections could form clusters containing over 25 worms. In a similar study by Franco, Huffman and Fried (1988) on the effects of worm crowding of adults of *E. revolutum* in experimentally infected golden hamsters, worms were recovered from both the small intestine and

the caecum. Hosier and Fried (1986) commented on the migrations of *E. revolutum* infections in both Swiss Webster (SW) and ICR mice and found that in both hosts worms excysted in the ileum and migrated anteriorly, away from the ileo-caecal valve. Simonsen and Andersen (1986), studying the dynamics of the antibody attack to the surface of *E. revolutum* in mice, recorded the presence of worms in the small intestine. Huffman, Alcaide and Fried (1988) commented on the alimentary tract positions of both *E. revolutum* and *E. liei* in single species infections and concurrent infections in experimentally infected golden hamsters while in an earlier study Huffman, Michos and Fried (1986), while investigating the impact of *E. revolutum* infections in the golden hamster, observed the presence of worms in the liver in acute infections.

The purpose of this section of the present study was to map the microhabitat utilization of *E. liei* in the alimentary canal of laboratory mice in experimental infections. This was done in a manner which enabled ontogenetic migrational activity to be surveyed and also provided data on worm aggregational behaviour in the host gut.

5.2 Materials and Methods

5.2.1 Basic infection and examination methods

Thirty-six, six week old laboratory outbred Swiss T.O. male mice were each infected with 25 metacercarial cysts of *E. liei* using standardised infection procedures (See Section 2.6). Another nine mice were each infected with 50 metacercarial cysts. Mice were killed at each of days 1 to 10 postinfection and then on days 15, 20 and 100 postinfection, three randomly chosen mouse replicates

being killed at each time interval. The nine mice infected with 50 metacercarial cysts were sacrificed on days 1 to 3 post-exposure and again three replicates were killed per day. At necropsy, mice were weighed and the small intestine removed, enabling the total length of the small intestine to be measured from the pyloric sphincter to the beginning of the caecum. The intestine was then cut into five sections of equal length and placed in 0.85% mammalian saline at 28°C. These gut sections were then opened longitudinally in a petri dish filled with saline and examined microscopically. Worms were not found using this technique in the first three days of infection in mice infected with 25 cysts, probably because of the small size of worms at this age.

In view of this, a different methodology was adopted for the density 50 infections. The intestine was removed and cut into five sections, each section was then opened longitudinally, pinned flat, and then flooded with aqueous Bouin's fixative (see Appendix 2) after which the small intestine was searched for fixed worms (small, young worms fixed in this way were easier to visualize). After the removal of the small intestine in the density 25 infections, the liver, spleen, stomach, kidneys, large intestine and bladder were removed and all these organs and the peritoneal cavity were searched for worms. The large intestine and stomach were both removed flooded with 0.85% saline and opened longitudinally and searched for worms. The liver, kidney, spleen and bladder were immersed in 0.85% mammalian saline, squashed and then examined after thirty minutes for the presence of worms. For the density 50 infections, the large intestine and stomach were removed flooded with Bouin's solution and then opened

longitudinally and the intestinal mucosa searched for worms. The liver, spleen, kidney and bladder were squashed in Bouin's solution and the remaining suspension searched for worms.

5.2.2 Measurement of worm positions

All worms that were located in the small intestine had their positions measured using a binocular microscope fitted with a previously calibrated graticule. The position measured was the distance from the pyloric sphincter to the site of attachment of the worms. The site of attachment, was assumed to be that of the ventral sucker. The positions of the worms once measured were converted to percentage distances along the small intestine from the pyloric sphincter to the intestinal-caecal junction. For the early worm infections (days 1 to 5) the mid-region of the body was taken as the attachment site as identification of the ventral sucker was not possible at the microscopical magnification utilized.

5.3 Results

5.3.1 Parasite spatial distribution in the host gut

E. liei was found to occur exclusively in the small intestine of laboratory-bred male Swiss T.O. mice. No extra-intestinal parasitization was exhibited by this parasitic digenean in this definitive host. Initial observations of the worms in the small intestine revealed that they were often massed together in aggregated clusters in the older infections, making it virtually impossible in some instances to discriminate the position of one worm from those of its immediate neighbours. Worms that were found to be attached to the small intestine were anchored firmly to

the mucosa by their ventral suckers which appeared to be the main attachment organ.

Qualitative observations suggested that worms in the older infections were usually to be found in large clumps, small groups or in pairs, with only a smaller proportion occurring in relative spatial isolation from their infection partners. In the earlier phases of infections, worms seemed less clumped and appeared to be more evenly dispersed spatially in relation to each other. The worms from 1 to 3 day old infections that were fixed in Bouins were found mainly in the posterior part of the small intestine. Data from the known age infections relating to the weight of the hosts, levels of worm establishment and mean longitudinal percentage positions of worms in the gut over successive days postinfection are summarized in Table 5.1.

Fig 5.1 shows the spatial distribution of *E. liei* in five longitudinal sections of the small intestine. The columns in each bar graph represent the percentage proportion of worms in each section of the intestine for all the replicates at each particular time interval. The individual histograms in Fig. 5.1 show that up to the time of sexual maturity, that is around 8 days postinfection, all worms are found in the posterior two sections of the intestines (from 60%-100% along its length). After 8 days there is evidence of anteriad movements of worm positions which take sexually mature worms into all sections of the intestine.

Fig. 5.2 graphically illustrates the positions of each individual worm monitored during the course of this experiment and along with

TABLE 5.1 **The recovery and mean position of E.liei in the small intestine at successive time intervals.**

Days Postinfection	Weight of Mouse (g)	No. of Worms recovered	Mean Worm Position (%)	No. of worms (n)	Standard Error (+/-) (of mean worm position)
1	35.00 36.20 37.40	12 9 7	90.30	28	0.84
2	36.00 36.00 34.70	17 12 8	84.90	37	1.06
3	38.40 32.10 34.00	16 10 21	86.20	47	0.67
4	36.30 37.80 34.80	7 12 9	83.80	28	2.53
5	33.30 35.70 35.80	12 11 18	86.60	41	0.54
6	35.50 35.40 39.50	9 22 17	81.30	48	1.06
7	36.60 35.30 35.00	9 11 16	74.90	36	0.44
8	41.30 40.30 36.20	11 10 12	77.10	33	0.94
9	34.00 35.20 36.70	5 25 14	60.80	44	1.76
10	37.20 30.50 36.80	11 24 13	66.00	48	1.12
15	41.20 35.60 35.50	18 9 22	69.00	49	2.31
20	40.10 36.10 36.30	21 7 6	57.10	34	3.30
100	38.30 41.30 42.10	23 14 12	38.10	49	0.63

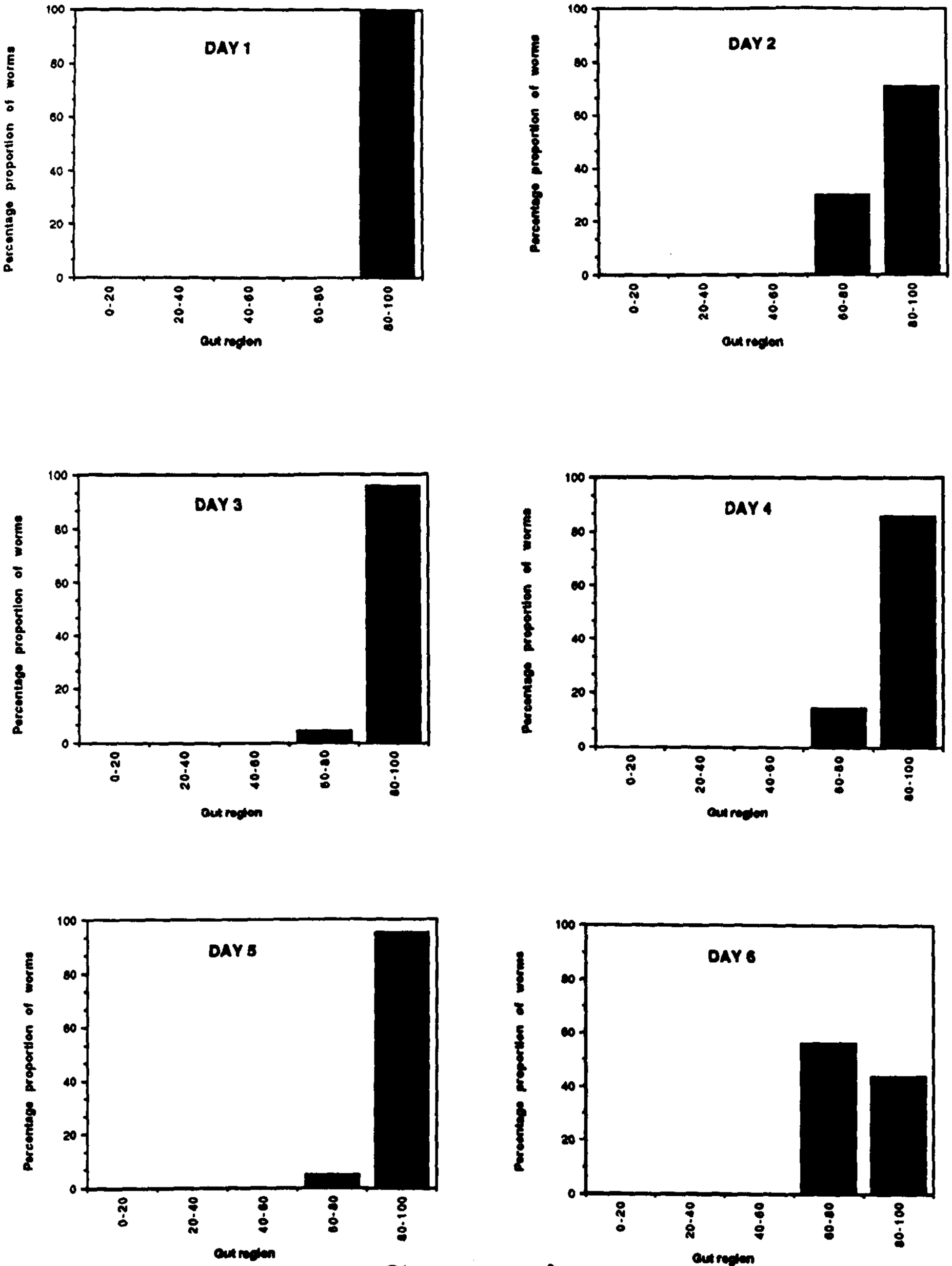
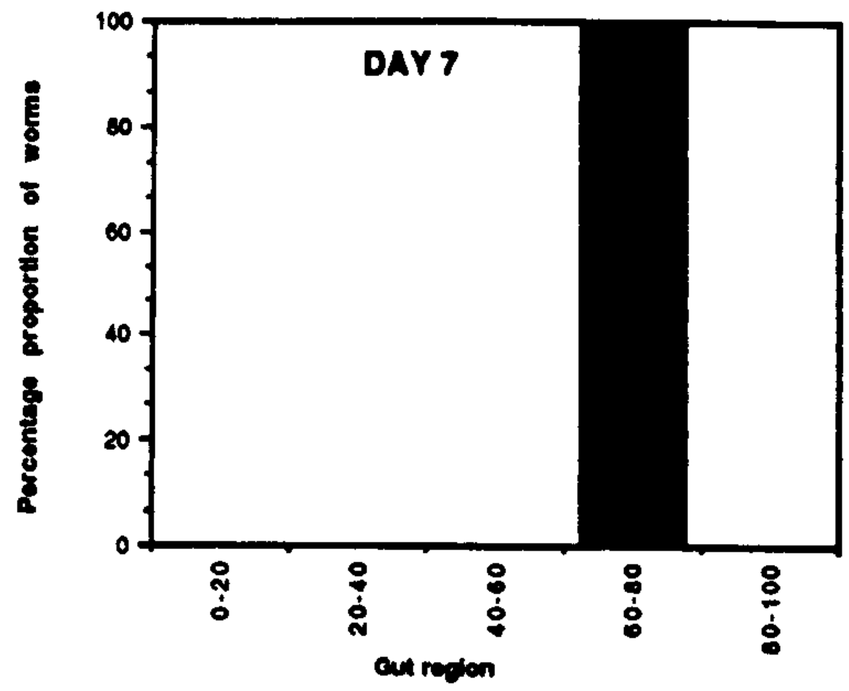
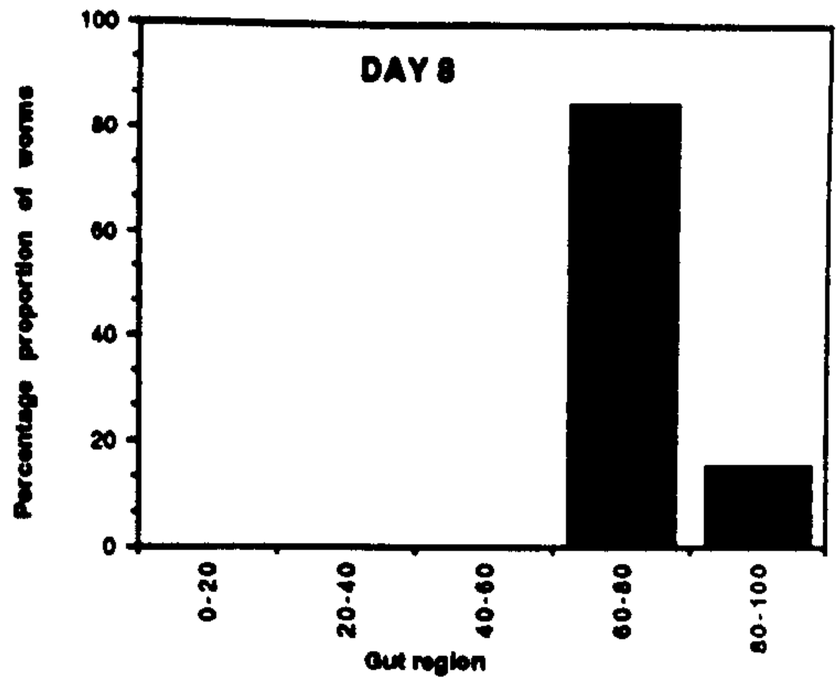
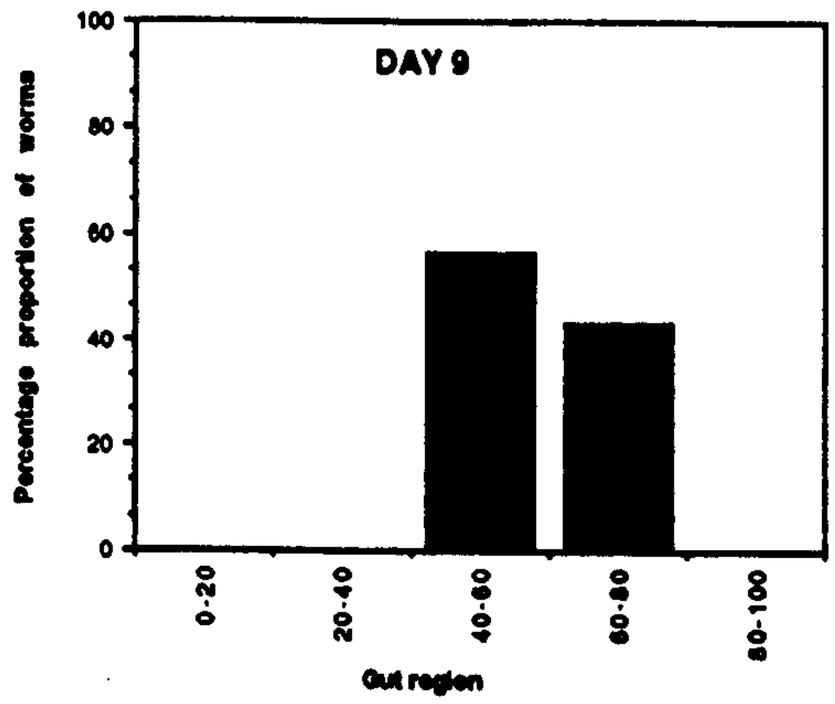


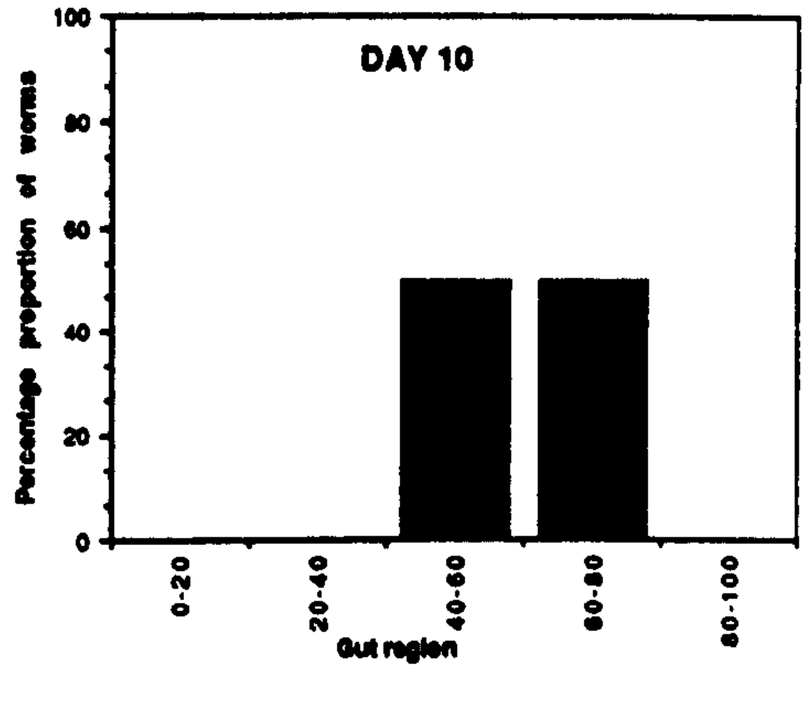
Fig. 5.1 The spatial distribution of *E. liei* in longitudinal sections of the small intestine

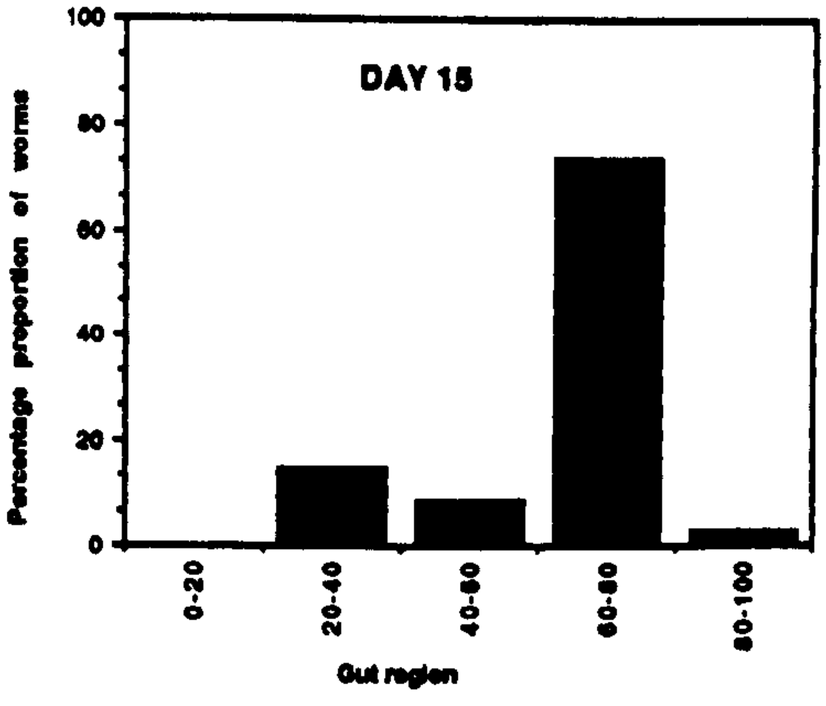
Percentage proportion of worms

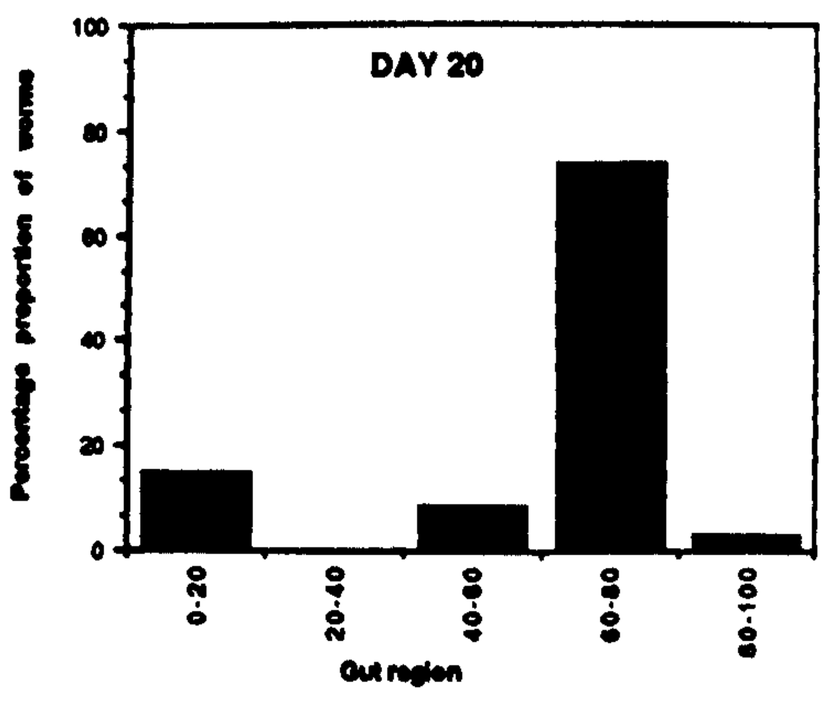












Gut regions

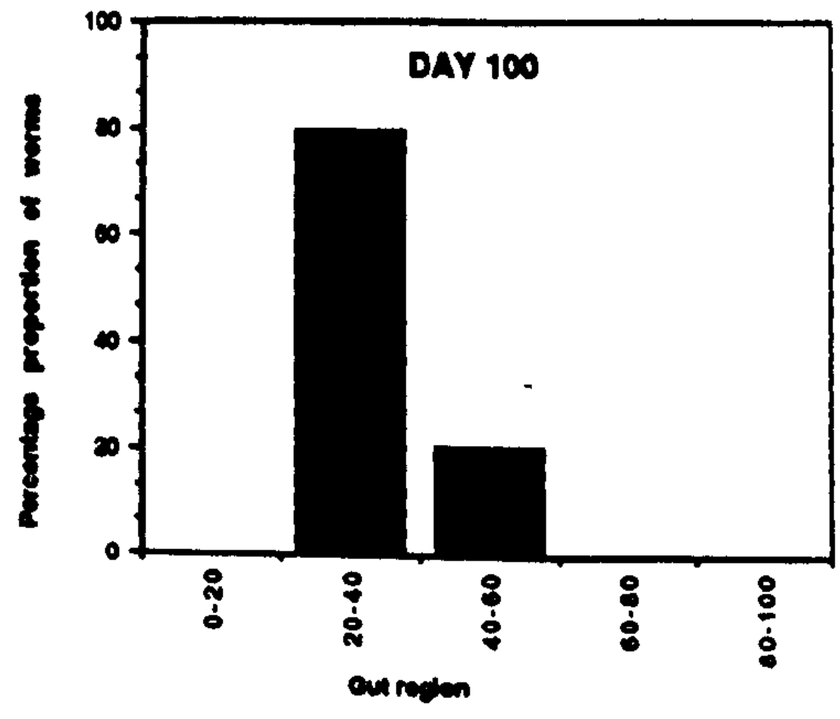


Fig. 5.2 The position of attachment of E. liei in the small intestine during the course of infection

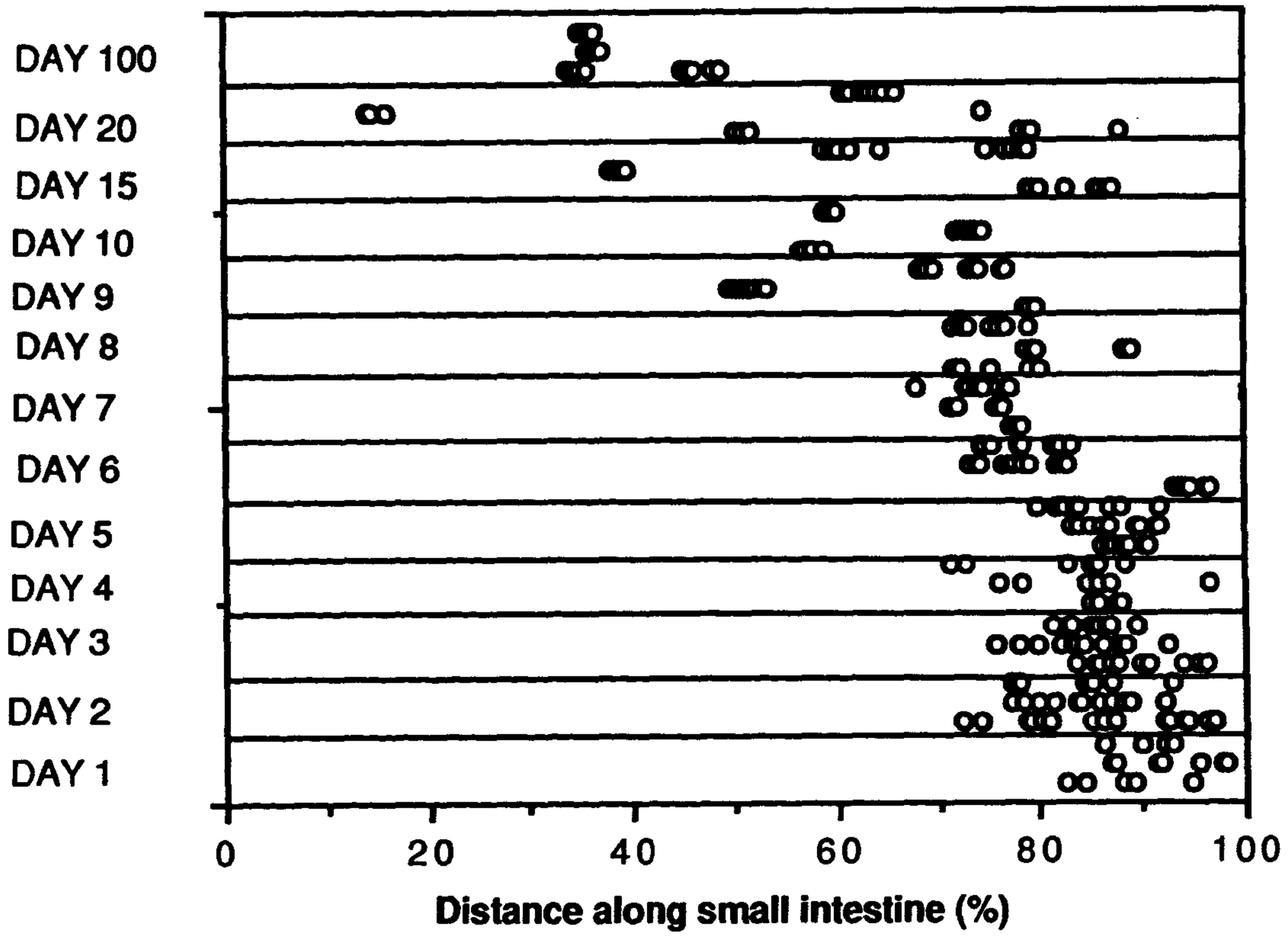


Table 5.1 and Fig. 5.1, provides a clear demonstration that there is a marked anterior movement by the worm populations between days 1 and 100 of an infection. There is direct evidence of an ontogenetic migration in this helminth. Fig. 5.3 shows the mean percentage location of the attachment sites of *E. liei* along the length of the mouse small intestine with standard errors of the means attached, at successive days postinfection. The mean position of the worm population decreases from 90.3% on day 1 to 38.1% on day 100. It appears that, as a synchronised infection ages the mean worm position moves anteriorly by a distance of approximately 50% of the intestinal length over 100 days. This represents an absolute movement of approximately 25 cm. Fig 5.3 reveals a relatively steady anterior movement of the mean worm position between day 1 and day 20. During this time the mean position alters in an approximately linear fashion between 1% and 2% per day. Thereafter, however, the change appears to slow down because between day 20 and day 100 the total change is only about 15% of the intestinal length representing a rate of change of only about 0.2% per day.

Fig. 5.3

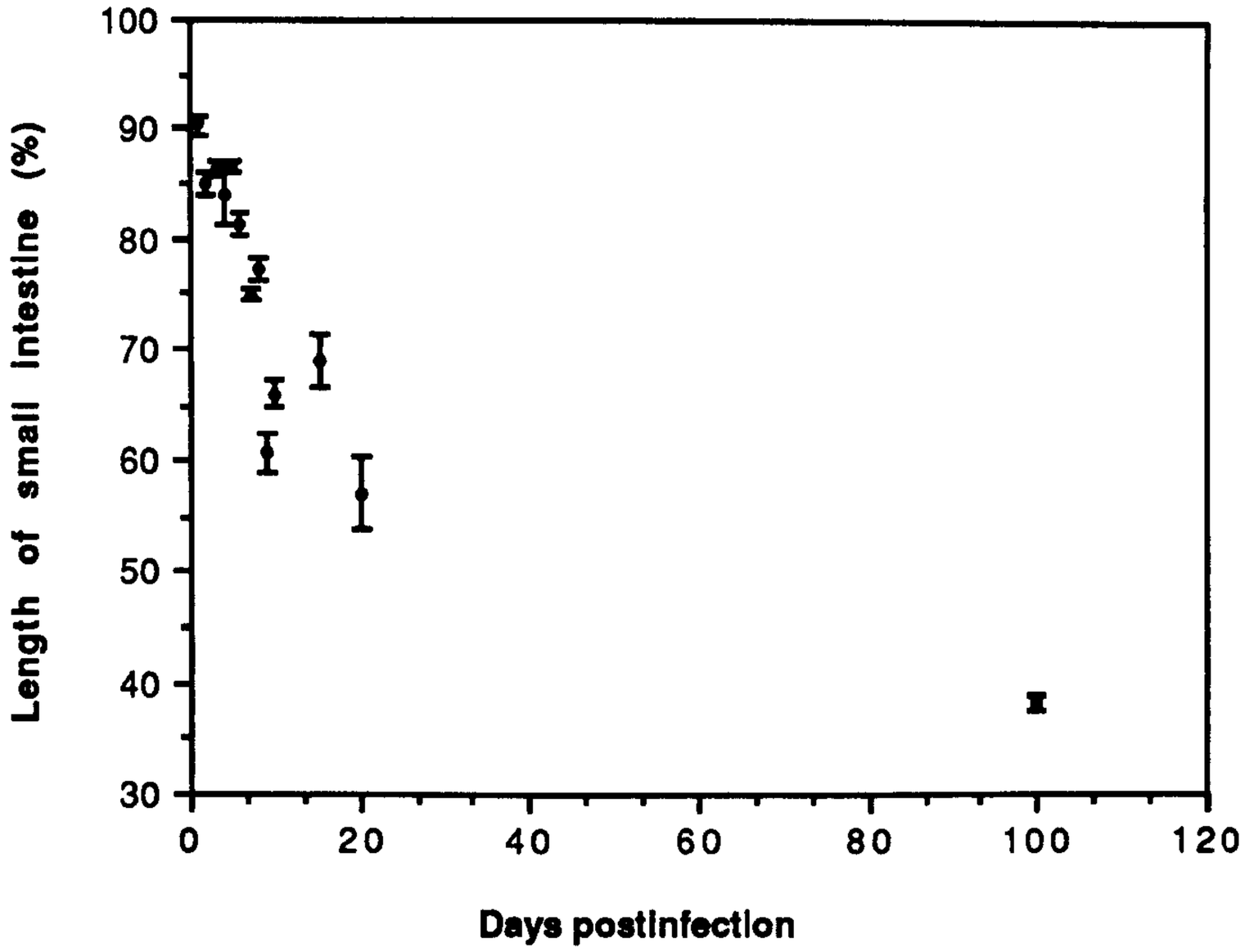


Fig. 5.3 The mean position of *E. liei* in the small intestine during the course of infection (data points include standard errors)

5.3.2 Nearest neighbour analysis

Direct examination of the appearance of worm populations in infected guts suggested that, at some phases of an infection, worms were highly aggregated spatially. To provide a more formal investigation of this aspect of the microhabit utilization of *E. liei* in the small intestine, a form of nearest-neighbour analysis was carried out. This was achieved by estimating the mean distance of each worm in a known-age infection to its nearest neighbour. As longitudinal locations along the gut had beenanalysed with respect to percentage positions (to the nearest 0.1% of the length of the intestine), this was the manner in which mean nearest neighbour spacings were expressed in the analysis. Worms that had initially been assigned the same longitudinal location were regarded as having a zero spacing from their nearest neighbour. Although this is only one possible way in which such individuals might have been treated analytically, the consistent use of this approach meant that mean nearest neighbour spacing could be meaningfully compared between one infection age and another. The results of such analysis are summarised in Table 5.2. The means of these nearest neighbour values for each time interval are represented graphically in Fig.5.4 with their standard errors of the means. The mean nearest neighbour values at day 1, 2, 3 and 4 are 3.86, 9.01, 3.74 and 4.15% respectively, values which contrast markedly with those of 0.15, 0.12, 0.86 and 0.06% for days 9, 10, 15 and 100. The large mean value of 7.86% for day 20 is due to single worms which are not in the vicinity of the main worm population which considerably increased the mean nearest neighbour spacing. The mean nearest neighbour spacing data (apart from this apparently altered situation on day 20), would appear to signify that as the infection

ages the value of the mean spacing of worms from their nearest neighbours decreases. This perhaps implies directed aggregation behaviour by the worms as the infection ages.

Fig. 5.4

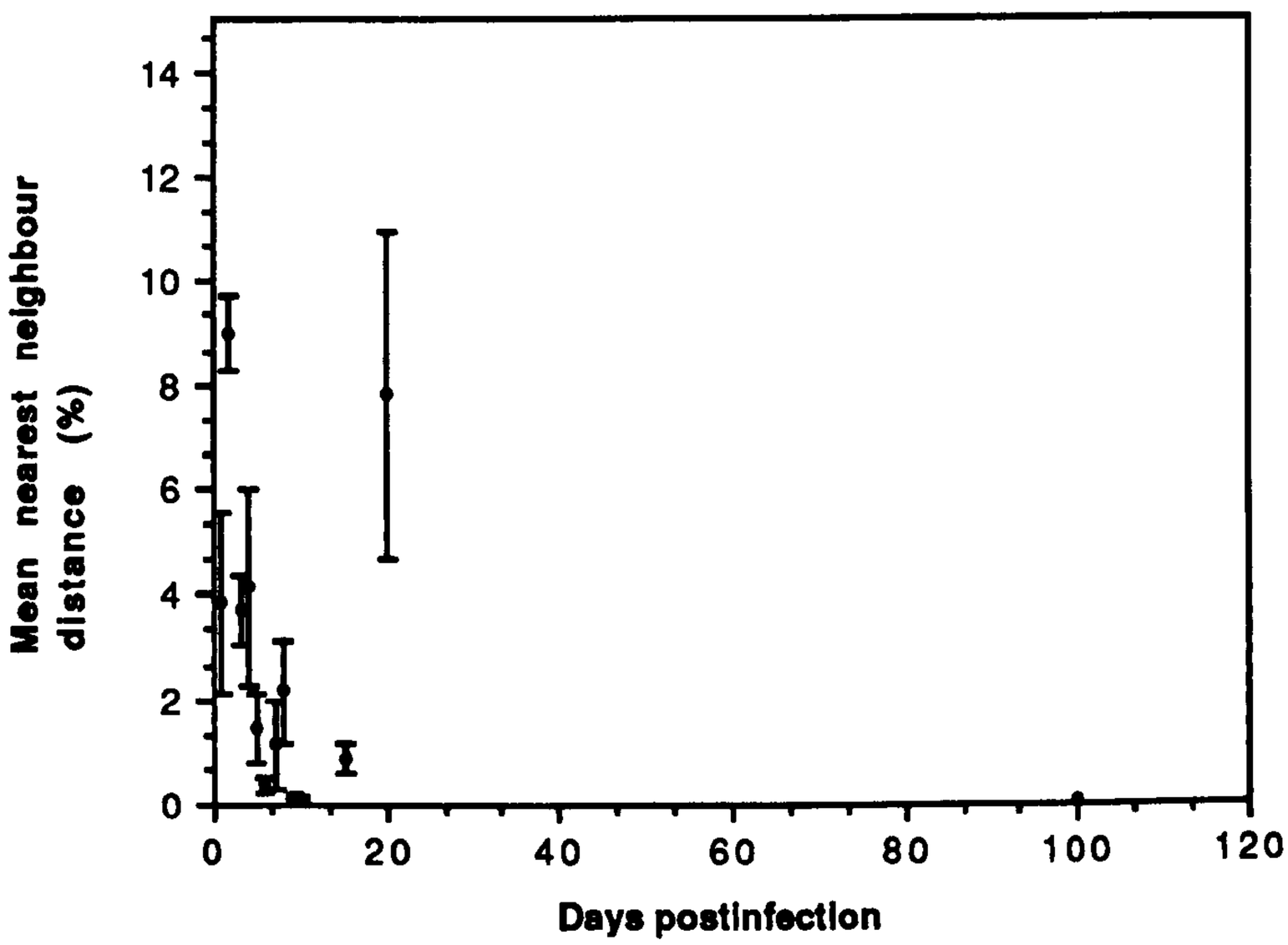


Fig. 5.4 The mean distance of *E. liei* to its nearest neighbour in the small intestine (data points include standard errors)

TABLE 5.2 **The nearest neighbour distance of E.ilei in the small intestine**

Days Postinfection	Nearest neighbour Distance (%)	Mean Nearest * Neighbour distance (%)	Standard error (+/-)
1	0.83 7.89 2.86	3.86	1.71
2	7.25 9.48 10.29	9.01	0.74
3	2.84 5.39 2.98	3.74	0.67
4	0.16 4.23 8.06	4.15	1.86
5	0.15 2.96 1.22	1.44	0.67
6	0.27 0.17 0.70	0.38	0.13
7	0.04 0.15 3.22	1.14	0.85
8	0.13 4.22 2.12	2.16	0.96
9	0.20 0.08 0.16	0.15	0.03
10	0.20 0.08 0.08	0.12	0.04
15	1.44 0.13 1.00	0.86	0.31
20	0.99 11.99 11.55	7.86	3.15
100	0.09 0.06 0.04	0.06	0.01

*n=3

5.4 Discussion

5.4.1 Microhabitat utilization and migration

Echinostomes are typically parasites of the intestines in both birds and mammals (Evans, 1983; Fried and Freeborne,1984; Christensen, Odaibo and Simonsen, 1988). It is evident from this report that, in Swiss T.O male mice, worms of *E. liei* exclusively occupy the small intestine and undergo a definite ontogenetic migration during the course of an infection. No evidence has been forthcoming using this host-parasite system to suggest that other sites may be utilized as has been demonstrated for *E. revolutum* in the hamster (Huffman *et al.*, 1986 and Franco, Huffman and Fried, 1988) where the use of liver and caecum sites have been noted.

The ontogenetic migration of *E. liei* that has been characterized in the course of this investigation moves the worm population in a anterior direction towards the stomach from a starting position near the rear end of the small intestine. No worms were ever recorded in more posterior gut regions such as the caecum, colon and rectum even on the first day of an infection. This absence suggests that metacercarial cyst activation and juvenile worm attachment occur exclusively in positions anterior to the ileo-caecal valve. If small numbers of attachments do occur very early in an infection area posterior to the valve, the worms concerned must be expelled or must themselves migrate very rapidly forwards to produce the results obtained here. The present findings relating to the ontogenetic migration of *E. liei* in Swiss T.O. mice are similar, in a general sense, to those of Hosier and Fried (1986) concerning SW and ICR mice infected with *E. revolutum*. These workers found that *E. revolutum* excysted mainly in the last 10 cm of the ileum of

these two strains of mice and that, during development, worms migrated anteriorly, gravid worms being recovered 20 to 30 cm anterior to the ileo-ceacal valve after an infection duration of four weeks. In the same study no worms were recovered from the caecum or colon.

A comprehensive study carried out by Gaillarde (1985) on the life cycle and development of *E. caproni*, considered the distribution of this echinostome in the small intestine of laboratory bred female mice. Gaillarde infected three groups of mice with 1 to 20, 25 to 50 and 50+ metacercarial cysts of *E. caproni* then sacrificed mice between days 43 and 52 postinfection. In the 1 to 20 density group, 73% of the worms were found between 50% and 70% along the length of the small intestine. In the 25 to 50 cyst density group, 54% of the worms were in the 50% to 70% region. In the third group, which were administered 50+ cysts, worms were present along most of the length of the intestine, 32% of the worm population were found between 50% and 80% but the anterior portion of the duodenum was uncolonized. Gaillarde noted in all the experimental groups the presence of dead worms in the caecum and colon. Gaillarde concluded that the preferred microhabitat of *E. caproni* for a population density approaching that found in natural infections is situated in the small intestine between 50% to 70%. This preferred region is similar to that demonstrated for sexually mature worms of *E. liei* between days 9 and 20 in the present study.

Fried and Freeborne (1984) noted, in chickens infected with *E. revolutum*, that as the worms aged they tended to move more

posteriad in the gut, a phenomenon recorded in *E. revolutum* in chicks in previous work carried out by Senger (1954), Fried and Weaver (1969) and Fried and Alenick (1981). Franco, Huffman and Fried (1988) studying the effects of worm crowding of *E. revolutum* in the golden hamster, observed that the spatial distribution of the parasites increased, that is, there was an increase in the total population spread along the gut as the cyst density increased. In low cyst levels worms were located in the jejunum but as the levels increased worms were found to be equally distributed in the duodenum, jejunum and ileum. Huffman, Alcaide and Fried (1988) studied single species and concurrent infections of the golden hamster with *E. revolutum* and *E. liei*. In single species infections, hamsters were fed 25 cysts of either *E. revolutum* or *E. liei* and in concurrent infections they were fed 20 cysts of *E. revolutum* and 20 cysts of *E. liei* simultaneously. These workers recovered worms from 7 to 21 days postinfection and divided the intestine into three sections; the duodenum, jejunum and ileum. In both types of single-species infection more than 60% of all worms from both species were recovered from the posterior third of the intestine, that is in the ileum. In *E. liei* infections, 88% of the worms were recovered from the ileum, the remaining 12% from the jejunum, while in *E. revolutum* infections, 65% of the worms were recovered from the ileum, 30% from the jejunum, and 5% from the duodenum. In concurrent infections, 41% of the *E. revolutum* were found in the ileum as were 80% of *E. liei*. In the jejunum were found 57% of the *E. revolutum* compared with 20% of *E. liei*. *E. liei* was not recovered from the duodenum in concurrent infections but 1.9% of the *E. revolutum* worms were found there. In this report no developmental migration of *E. liei* was noted. These workers

did suggest that the apparent anterior migrations of *E. revolutum* in concurrent infections occurred to reduce its overlap with the *E. liei* population. Odaibo, Christensen and Ukoli (1988) examined the distribution of *E. caproni* in the small intestine of NMRI mice infected with 6 and 25 metacercarial cysts and noted the presence of the worms from 20% to 100% along the length of the intestine up to 24 hours after exposure. They stated that worms migrated posteriorly and established in the 60% to 100% region on days 3 and 7. In the 25 cyst infection, worms remained in the 60% to 100% up to 42 days following infection. In contrast, they found that in infections with 6 metacercariae worms migrated anteriorly with the worms establishing in the 20% to 80% region on days 14 and 21. These workers did not discuss possible reasons for these migrations or comment on the different migration patterns observed.

Huffman *et al.* (1986) studied the pathological effects of *E. revolutum* infection in golden hamsters and revealed that *E. revolutum* migrated from the small intestine to the liver in this host. This migration was only noted in acute heavy infections, that is, hamsters infected with 300 metacercarial cysts simultaneously and they speculated that the route taken by *E. revolutum* was via the common bile duct. They believed this migration was due to either *E. revolutum* being present in an unnatural experimental host or because of the worm crowding. Huffman *et al.* (1988) concluded that the migratory patterns of *E. revolutum* and *E. liei* differ markedly in that *E. liei* does not migrate into the common bile duct.

Although ontogenetic migrations of endoparasitic helminths are apparently of common occurrence, very little is known about the selective advantages associated with them or the stimuli that bring them about. Some of the major advantages of the anterior migrations of *E. liei* are probably related to the maintenance of the parasite in the optimal micro-habitat for existence and reproduction in the small intestine. The anterior migrations of the cestode *Hymenolepis diminuta* have been demonstrated by Turton, (1971) and Braten and Hopkins (1969). Braten and Hopkins transplanted young worms into the posterior ileum of infected rats and observed a marked anterior migration of the worms to the correct position against the direction of gut peristalsis. Crompton and Whitfield, (1968) postulated that the anterior migrations of *H. diminuta* and *Moniliformis dubius* are effective in maintaining the maximum proportion of worm body surfaces within the optimum region of the small intestine in a manner which compensates for the linear growth of the worms. In *H. diminuta* the ontogenetic migration coexists with a feeding migration documented by Mettrick, (1971). Mettrick showed that there is a posterior migration of the worms during the day in the intestine and an anterior migration by night coinciding with feeding by night and fasting by day of the rat host. This observation implies that parasite feeding migration is a result of an external behavioural pattern on the part of the definitive host. Chappell (1979) considered that this type of migration would occur in parasites not firmly attached to the intestine. The anterior migrations of *E. liei* are probably related to the selection of optimum sites for development, feeding and reproduction along one or more of the

many gradients along the length of the intestine (Read, 1971; Evans, Pye, Bramley, Clark, Dyson and Hardcastle, 1988).

Crompton (1973) reviewed the sites occupied by a number of parasitic helminths in the alimentary tracts of their vertebrate hosts and concluded that most species of adult digeneans appear to be restricted to the paramucosal lumen and the mucosal and epithelial tissues of the gut. He suggested that their limited radial distribution may be related to the fact that species have become adapted to feeding on the mucosa and materials associated with it. These preferred microhabitat sites may also be assumed to be related to parasite feeding, development and reproduction. Fried and Diaz (1987) looked at site selection and site finding behaviour of 14-day old *E. revolutum* originally grown in domestic chicks. This was studied by inoculating single worms into various sites on the chorioallantoic membrane of 13-day-old chicks embryos. Their findings suggested that *E. revolutum*, regardless of the site of inoculation preferred the chorioallantoic membrane above the embryo. They gave no reasons for this site preference but speculated on the possible involvement of chemoattractants released from the embryo.

The initial site selection exhibited by *E. liei* in Swiss T.O. mice as demonstrated in this study, is probably related to the essential endogenous and exogenous processes that intiate and sustain excystation in the small intestine. It is in this particular environment that the necessary extrinsic factors which may be involved in excystation, are present. It may be supposed that stimulus conditions related to pH, digestive enzymes, bile salts and

the presence of carbon dioxide (Smyth and Halton, 1983) initiate the excystation process within the gut anterior to the ileo-caecal valve. An interaction of these stimuli and the typical rate of passage of cysts along the alimentary tract could result in most initial attachment occurring in the posterior sections (the last 20%) of the ileum as shown in this study. A similar excystation and establishment pattern has been demonstrated by Fried and Kletkewicz (1987) who reported on the excystation of *E. revolutum* metacercariae in the domestic chick and concluded that excystation could occur within 0.5 hours in the lower ileum, this region being a preferred site for the attachment of *E. revolutum* in this host. *In vitro* excystation of metacercariae of *E. liei* has been carried out previously by Fried and Emili (1987, 1988) the process in the latter investigation being carried out in an alkaline bile-trypsin medium at 41°C. These are certainly some of the conditions similar to those that cysts would encounter within the small intestine of Swiss T.O. mice.

Very little is known about the sensory and effector mechanisms by which digeneans select sites within their hosts (Ulmer, 1971). Sensory receptors in adult digeneans have been studied by a number of workers. Morris and Threadgold (1967) considered sensillae of *Fasciola* adults to be rheoreceptors, used in detecting the direction of flow in a fluid medium. Erasmus (1967), in a study of the strigeid *Cyathocotyle bushiensis*, considered that concentrations of sensillae on lobes of the holdfast facilitated the attachment of the adult to the host mucosa. Smales and Blankespoor (1984) observed papillae on the tegumental surfaces of both *E. revolutum* and *Isthmiophora melis* and speculated

about their possible roles in monitoring attributes of the host internal environment. Danley (1973) and Fried and Bradford (1984) have provided evidence that secretions from the Harderian gland attract *Philophthalmus megalurus* and chemoattractants from the bursa of Fabricius attract *Leucochloridiomorpha constantiae* respectively to these preferred sites.

There is indirect evidence in the present study that the microhabitat utilization of *E. liei* is generated by active directed movements of the worms, that is, it is a consequence of active site selection. This is particularly clearly demonstrated by a careful examination of the relationship between establishment success and longitudinal location during the anteriad ontogenetic migration. Fig. 5.3, for instance shows that many worms must move out of their initial attachment area to account for the expansion in the range of gut locations utilized. The pattern of change could not be produced by, for instance, differential mortality of worms in different regions after a more widespread initial attachment. Indeed, Table 5.1 reveals that there is very little effective worm loss from the population during the period from 1 to 100 days after infection, there being no significant deviation from a mean establishment level of about 52% throughout this period. It appears that most "mortality" during this period occurs as unsuccessful establishment with cysts or juvenile worms being passed out of the gut before the first examination period (24 hours postinfection).

Other work on digeneans has provided similar, clear evidence for a directed change in location rather than differential mortality. Radlett (1979), for instance, observed the excystation of *N.*

attenuatus in the lower intestine of the domestic fowl and suggested that the worms subsequently actively migrate into the caeca. He stressed that the worms were not carried to this position by peristaltic activity but was unable to explain what stimulus caused the movement of newly excysted worms to the caeca, hypothesising that the differences in type and consistency of contents of the gut regions might play a part in generating this stimulus. Worms were recovered from the caeca after 4 hours, after which they migrated down the caeca, finally residing in their blind distal regions as they approached maturity.

5.4.2 Aggregation behaviour

E. liei infections in the small intestine appear initially to exhibit a relatively evenly dispersed spatial distribution in the posterior 25% of the small intestine but as the infection ages, worms migrate to form aggregated clumps. Nearest neighbour analysis provides a description of this phenomenon and shows that the mean spacing of each worm from its nearest neighbour decreases as the infection ages despite the fact that a larger and larger segment of the whole gut is being utilized during this period.

E. liei then, shows a tendency to form clusters within the mouse intestine as the infection ages. This type of clustering process has been noted by Fried and his co-workers with respect to *E. revolutum* (Fried and Alenick, 1981; Fried and Freeborne, 1984; Franco *et al.*, 1988) and other digeneans such as *Zygocotyle lunata* in the intestines of chicks (Fried and Nelson, 1978) and *Philophthalmus gralli* grown in chickens eyes (Nollen, 1983). *In vitro* studies have shown the tendency of *E. revolutum* (Fried,

Tancer and Fleming, 1980; Fried and Wilson, 1981; Fried and Pallone, 1984) and other digenean parasites such as *Leucochloridiomorpha constantiae* (Fried and Roberts, 1972) and *Fascioloides magna* (Foreyt, Samuel and Todd, 1977) to "pair". Interspecific "pairing" has also been observed by Fried and Jacobs, (1980) who concluded that *E. revolutum* is capable of considerable interspecific "pairing" with other species of hermaphroditic digeneans. A later study by Fried and Haseeb (1990) showed the ability of *E. caproni* and *E. trivolvis* adults to "pair" *in vitro* both intra-and interspecifically.

Fried (1986) concluded, after reviewing chemical communication in hermaphroditic digenetic trematodes, that monoecious digeneans "pair" or aggregate both *in vivo* and *vitro*. It must be pointed out that by "pairing" Fried does not imply copulation but the coming together and contact of two individual worms. In the studies carried out by Fried and Pallone (1984), Fried, Tancer and Fleming (1980) and Fried and Roberts (1972) no copulatory behavior was observed. Thin layer chromatographic studies have indicated that free sterols are involved in intraspecific "pairing" of excysted *L. constantiae* metacercariae and *E. revolutum* adults (Fried and Gioscia, 1976 and Fried *et al.* 1980). This led Bennett and Fried, (1983) to state that the sterol fraction of the excretory-secretory products of *E. revolutum* adults acts as a chemoattractant. Their thin layer chromatographic analyses showed that cholesterol was the only free sterol present in the excretory-secretory products of 7 to 21 day old worms of *E. revolutum*. Fried *et al.*, 1980 noted that "pairing" of adult worms was highly significant but that chemically excysted metacercariae did not "pair". With this in mind,

it is interesting to note that Barrett, Cain, and Fairbairn (1970) who identified sterols in *Ascaris lumbricoides*, (Nematoda) *Macracanthorhynchus hirudinaceus*, *Moniliformis dubius* (Acanthocephala) and *E. revolutum* and observed their inability to synthesize such molecules, suggest that intestinal parasites obtain cholesterol only from the host diet or host secretions.

Fried (1986), has suggested that digenean aggregations may confer nutritive, developmental and reproductive advantages. All of these may be operating to account for the aggregational behaviour of *E. liei* noted in this study. The fact that clustering increases with age and is maximal after sexual maturity certainly suggests that reproductive advantages may be important. By analogy wth other echinostome studies it seems possible that *E. liei* is releasing sterols as chemoattractants. If so, they would be operating in a pheromone-like way (Bone, 1982).

CHAPTER 6

PARASITE POPULATION DENSITY-RELATED EFFECTS IN *ECHINOSTOMA LIEI* INFECTIONS IN MICE AND THE PATHOGENESIS ASSOCIATED WITH INFECTION

6.1 Introduction

The growth, development and fecundity of *E. liei* in definitive hosts and its pathological effects with respect to increasing worm intensities have not received the same attention that these topics have with respect to other echinostomatid digeneans. Some of these aspects have been investigated in *E. revolutum* in hamsters and chicks (Franco, Huffman and Fried, 1988; Fried and Freeborne, 1984 respectively), in *E. caproni* in NMRI mice (Odaibo, Christensen and Ukoli, 1988) and in *E. malayanum* in male albino rats (Mohandas and Nadakal, 1978). Parts of these density-related experimental studies have concentrated on the effects of what was characterised as intraspecific crowding of worms. This phenomenon was apparently confirmed in *E. malayanum* infections in rats by Mohandas and Nadakal (1978). These workers attributed the diminution in size of worms in high density infections to this type of interaction. Franco *et al.* (1988) noticed similar effects of crowding with *E. revolutum* infections in golden hamsters.

This phenomenon has also been observed in other digenetic systems. Nollen (1983) observed a similar effect of crowding on *Philophthalmus gralli* grown in chickens as did Boray (1969) in *Fasciola hepatica* in mice. The phrase "crowding effect" has come to denote the usually deleterious effect of increased worm burdens on size, establishment and developmental rates of parasitic helminth infections.

The population consequences of crowding on size, establishment and developmental rates of non-digenean parasitic infections

have been assessed by Jarrett, Jarrett and Urqu hat, (1968) and Halvorsen and Andersen (1974) and reviewed by Keymer (1982). Jarrett *et al.*, (1968) examined populations of the nematode *Nippostrongylus brasiliensis* in the rat and noticed reductions in both size and establishment in large populations, while Halvarsen and Anderson (1974) observed the effects of parasite population density in infections of the tapeworm *Diphyllibothrium dentriticum* in the golden hamster and observed a reduction in size of the tapeworms in denser populations. Similarly, Roberts (1961) has shown that when *Hymenolepis diminuta* is grown in rats there emerges a clear relationship between the intensity of infection and a reduction in growth, maximum worm weight and length and egg production. A later study by Hesselberg and Andreassen (1975) also clearly showed that the length, weight and egg production of *Hymenolepis diminuta* decreased with increasing population density.

Keymer (1982) commented that these density-dependent mechanisms are considered to be important in stabilizing the population growth in both free living and parasitic organisms. Christensen, Odaibo and Simonsen, (1988) pronounced that *Echinostoma* population regulation in rodent experimental hosts is governed by the host's capacity to express an effective regulatory, presumably immunological, response, while Bradley (1972) suggested that the regulation of parasitic populations is linked with density-dependent control of within-host parasite numbers. He pointed out that host-parasite interplay with respect to parasite establishment, survival and fecundity are

affected by both host-and parasite-related factors. With regards to host-related factors, these include such attributes as species, strain and age, while parasite-related factors that enter the interplay include species, age and the intensity of worm infection.

Even within the restricted area of studies on echinostomes in small mammals, however, there is no clear consensus with respect to the importance of parasite population density-related effects. Mohandas and Nadakal (1978), using *E. malayanum* infections in rats, for instance, observed that the density-dependent effects on the reproductive capacity of individual worms occurred only at very high worm burdens. In contrast Odaibo, Christensen and Ukoli (1988) found no density-dependent constraints on the fecundity of *E. caproni* in NMRI mice, although density-dependent constraints on fecundity have been identified and described in other helminth/host combinations. Such studies include that of Kino (1984) who examined the effects of parasite density on *Angiostrongylus cantonensis* in rats and Anderson and Schad (1985) who studied the impact of mixed *Necator americanus* and *Anclyostoma duodenale* worm burdens in human infections. Scott and Lewis (1987) reviewing the population dynamics of helminths in wild and laboratory rodents stated that parasite density has an effect on the ability of individual parasites to grow, reproduce and survive in the definitive host while also influencing the ability of infected hosts to survive and reproduce.

Descriptions of the pathology associated with ali mentary tract echinostomiasis include a number of case histories of human infections with parasites such as *E. malayanum* (Lie and Virik, 1963) and *E. ilocanum* (Cross and Basaca-Sevilla,1986). Yamashita (1964) explained that the effects of echinostomiasis in man were closely related to worm burden. The clinical and pathological effects of *Echinostoma* in rodent hosts has been examined by Mohandas and Nadakal (1978), Huffman Michos and Fried (1986) and Huffman, Alcaide and Fried (1988). Other histopathological studies of *Echinostoma* infections include those on *E. malayanum* in pigs (Haque and Siddiqui, 1978), *E. revolutum* in domestic ducks (Kishore and Sinha, 1982), *E. revolutum* in conventional mice and congenitally athymic nude mice (Bindseil and Christensen, 1984) and *E. caproni* in chicks (Kim and Stuart, 1989). In all of these cases *Echinostoma* infection has been shown to lead to identifiable damage to the small intestine, particularly erosion of the intestinal villi.

The objective of this component of the present research programme was to assess the effects of various worm densities of *E. liei* on the establishment success, gut distribution, size, *in-utero* egg counts and egg output of the parasites themselves as well as on the weight and levels of anaemia in the mouse host. A principal objective was also to describe the main pathological features associated with *E. liei* infections in laboratory mice.

6.2 Materials and Methods

6.2.1 Initial infection and faecal analysis

Three sets of five, 6 week old laboratory mice were administered either 6, 25 and 100 metacercarial cysts of *E. liei* following a standardized procedure (see Section 2.7). One mouse from each cyst density set was then killed on days 8,12,16,20 and 24 consecutively. Prior to the administering of cysts, mice were weighed and then they were weighed subsequently on each day postinfection throughout the duration of the experiment. Additional uninfected controls consisted of 5 mice, one of which was killed on each of the aforementioned days of the experiment. The day before mice were killed, they were placed for 24 hours in bottomless cages, containing saline in a lower reservoir, so that their faecal output over this time period was collected (see Section 2.7.1). From each 24 hour faecal sample, two sub-samples were taken and the mean number of eggs in that 24 hour faecal output was estimated (see Section 2.7.2). Mice were then weighed for the final time before blood samples were taken.

6.2.2 Haematological investigations

6.2.2a Haematocrit value

Blood samples, taken from the hosts' tails were prepared by using heparinized capillary tubes (Gelman and Hawksley Ltd.) to determine the packed cell volume (PCV or Haematocrit value). The heparinized tubes were 75 mm in length and had an internal diameter of approximately 1 mm. The blood was allowed to enter the tubes by capillary action, leaving one end unfilled (approximately 5 mm). This unfilled section was then

filled with critoseal (Gelman and Hawksley Ltd.), to seal the tube completely at one end. Two samples of blood were taken from each mouse and spun immediately in a microhaematocrit centrifuge (Gelman and Hawksley Ltd). The haematocrit tubes were spun for 5 minutes at 10,000 rpm to pack the red cells to a constant volume. To estimate the PCV the ratio of red-cell column to the whole column, (that is red cells plus plasma) in the haematocrit tubes was calculated. The mean value of the two blood samples was taken as the final PCV value.

6.2.2b Differential leucocyte counts

Blood smears were taken from each mouse at each time interval postinfection to carry out differential leucocyte counts. Blood smears of the mouse host were made from tail vein preparations to obtain thin films of blood spread on glass slides. To accomplish this a drop of blood was placed on a clean slide and the smear made using the technique described by Dacie and Lewis (1984). These thin films were then allowed to air-dry after which they were fixed in methanol for 5 minutes. Prior to staining, slides were washed briefly in tap-water and then placed in a dish containing 10 mls of buffered distilled water (see Appendix 1) so that the slides were completely covered. These slides were then stained for 30 minutes by adding 5 mls of Giemsa stain (see Appendix 1). The stained blood smears were then washed briefly in tap water to remove excess stain and allowed to dry in air. Blood smears prepared in this way were then viewed under oil immersion at x1000, using an Olympus Vanox AH-2 microscope. The longitudinal method (Dacie and Lewis, 1984) of performing differential leucocyte

counts was carried out. This technique involved the counting of a 100 leucocytes in a longitudinal strip on each slide. The 3 cell types counted were (i) lymphocytes, (ii) monocytes and (iii) the blood cells of the granulocytic series, that is eosinophils, basophils and neutrophils. Basophils are known to be very rarely seen in mouse blood preparations (Gude, Cosgrove and Hirsch, 1982) and because of the considerable variation in nuclear morphology, size of granules and intensity of cytoplasmic staining of the eosinophils and neutrophils with Giemsa, these three cell types were grouped together as the granulocytic series.

6.2.3 Necropsy

At necropsy the small intestine was removed and measured from the pylorus to the caecum, cut into five equal-length sections and each section opened longitudinally in 0.85% saline at 28°C. Worm positions were measured as described in Section 5.2.2 and the worms were then removed from the intestine. The associated organs of the peritoneal cavity were also examined for worms. The uteri of each worm, from a random sub-sample from each density set, was then teased open and the number of eggs in the uterus counted. For body measurements worms were fixed for 15 seconds in Berlands solution (see Appendix 1) and then stored in 70% alcohol. A sub-sample from each density set had their maximum lengths and widths measured with a pre-calibrated eyepiece micrometer after fixation.

6.2.4 Histological preparations

In infected mice, histological examination was made of the changes evident at the site of attachment of the main cluster of the worms to the mucosa in the small intestine and at sites adjacent to these clusters. Sections of the intestine were removed and fixed immediately in formol acetic-acid (FAA) (see Appendix 1). Corresponding areas of the small intestine in control mice, were removed and fixed in the same way. These infected and uninfected tissue samples were then processed using routine histological techniques, embedded in wax and sectioned at 6 micrometres and stained in haematoxylin and eosin (see Appendix 1).

6.3 Results

6.3.1 Worm establishment

The establishment success of *E. liei* following various metacercarial cyst exposure densities was measured by assessing worm establishment at different times postinfection. These results from this non-replicated material are summarized in Table 6.1. Fig. 6.1 represents these results graphically with the percentage establishment value plotted against the initial metacercarial cyst density. Absolute numerical worm establishment as measured over the period from day 8 to 24 increased as the metacercarial cyst exposure density increased (see Fig. 6.1) but analysis of the results showed that with increasing metacercarial cyst densities the percentage of cysts that successfully established was declining significantly ($N=15$; $R=0.66$; $P<0.01$, see Fig. 6.1).

6.3.2 Worm locations

The mean percentage positions of *E. liei* along the mouse small intestine at each successive time interval are described in Table 6.2 as the mean position of worm attachment in each of the worm populations. At day 8 the worms appear to occupy very similar mean positions in the small intestine, (with values of 79.9%, 77% and 75.5%) after initial metacercarial cyst exposure densities of 6, 25 and 100 respectively. For each metacercarial cyst density over the period from 8 to 24 days postinfection there appears to be an anterior movement of the worms as is evident in Fig 6.2 which shows the positions of the worms over this time period along with the standard errors of the mean values.

To test if there was any real significant difference between the mean positions of worm populations resulting from different cyst exposure densities on different days postinfection, the single factor analysis of variance test was carried out. This test was applied from day 16, onwards (that is after the initial ontogenetic migration) and the percentage position data was arcsin transformed as recommended by Schelfer (1979). On each day studied, a very significant difference in mean position was apparent in the different mice.

TABLE 6.1 The percentage establishment success of E. liei at various cyst exposure densities

Days postinfection	Establishment					
	Density 6		Density 25		Density 100	
	No.	%	No.	%	No.	%
8	5	83	15	60	28	28
12	5	83	10	40	33	33
16	2	33	16	64	24	24
20	5	83	7	28	32	32
24	4	67	14	56	50	50

TABLE 6.2 The mean percent positions of E.liei in the small intestine at various cyst exposure densities

Days postinfection	Mean gut positions (%)		
	Density 6	Density 25	Density 100
8	79.90 (+/-0.63)* n=5	77.00 (+/-1.03) n=15	75.50 (+/-0.67) n=28
12	66.00 (+/-0.59) n=5	58.80 (+/-0.34) n=10	67.50 (+/-0.28) n=33
16	71.70 (+/-1.41) n=2	62.50 (+/-1.19) n=16	53.10 (+/-1.63) n=24
20	65.30 (+/-2.93) n=5	36.70 (+/-0.23) n=7	59.70 (+/-0.70) n=32
24	62.40 (+/-0.16) n=4	70.50 (+/-1.26) n=14	59.50 (+/-1.06) n=50

*Figures in brackets refer to standard error of the mean values

TABLE 6.3 The percentage proportion of worms in gut sections of the small intestine at various cyst exposure densities

Gut Sections-%	Density 6				
	Day 8	Day 12	Day 16	Day 20	Day 24
0-20	0	0	0	0	0
20-40	0	0	0	0	0
40-60	0	0	50	40	0
60-80	40	100	50	60	100
80-100	60	0	0	0	0
	Density 25				
	Day 8	Day 12	Day 16	Day 20	Day 24
0-20	0	0	0	0	0
20-40	0	0	0	100	0
40-60	0	70	25	0	0
60-80	73.3	30	75	0	100
80-100	26.7	0	0	0	0
	Density 100				
	Day8	Day12	Day 16	Day 20	Day 24
0-20	0	0	0	0	0
20-40	0	0	8.3	0	0
40-60	0	0	87	40.6	42
60-80	73.3	100	4.7	59.4	58
80-100	26.7	0	0	0	0

Fig. 6.1 Establishment success at various cyst exposure densities

Fig. 6.2 Mean position in the small intestine after various cyst exposure densities (data points include standard errors)

Fig. 6.1

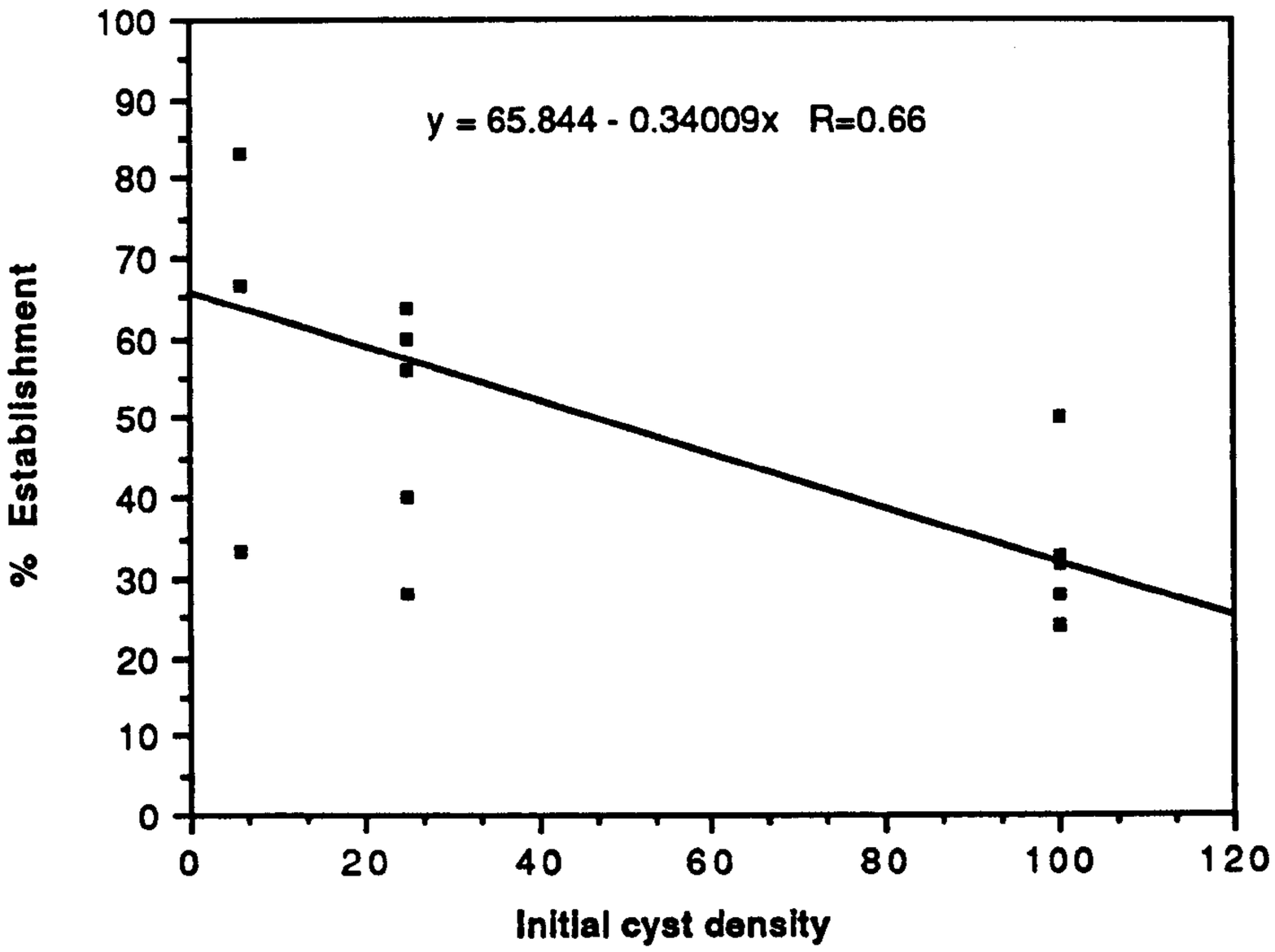
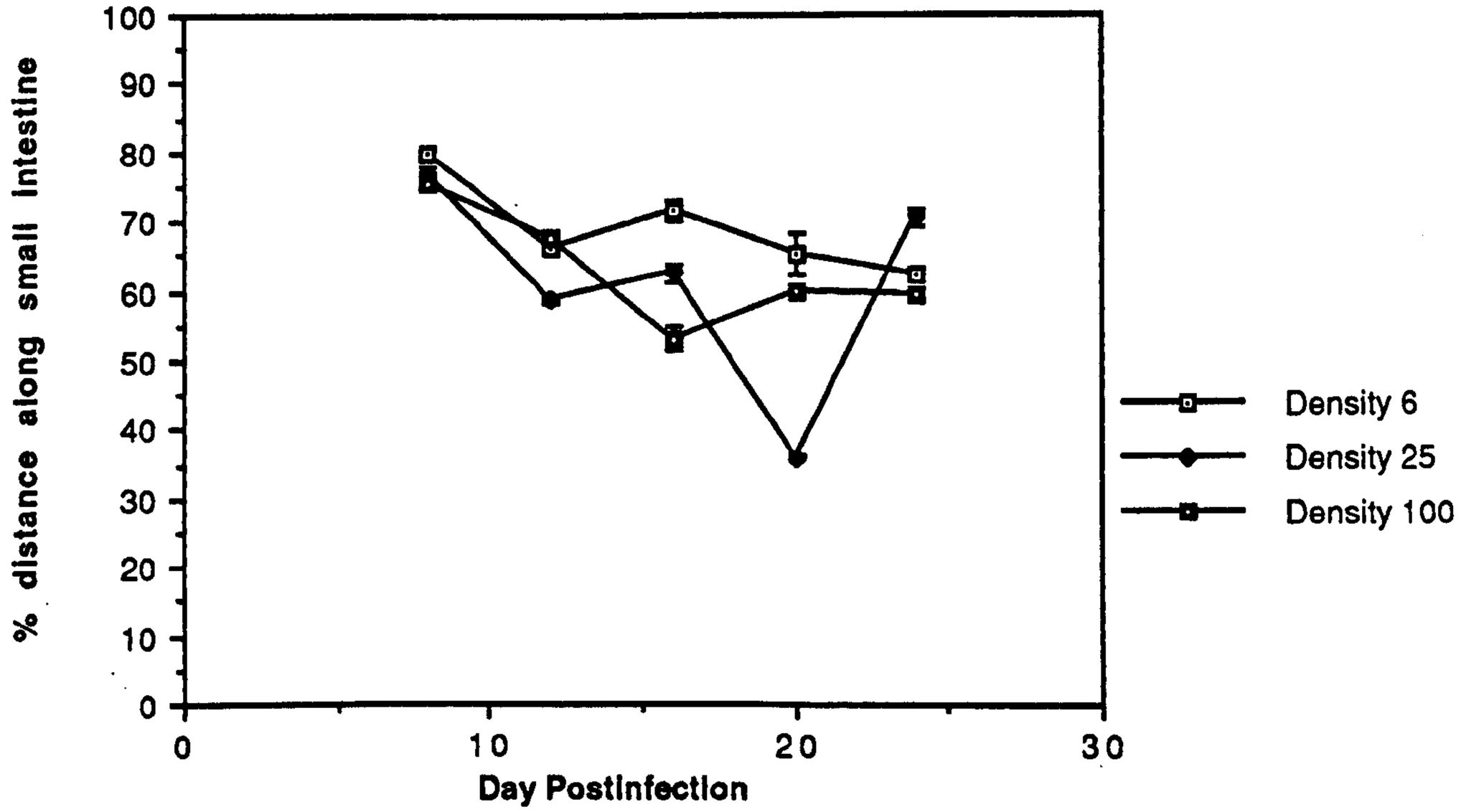

y = 65.844 - 0.34009x R=0.66
% Establishment
Initial cyst density
0
10
20
30
40
50
60
70
80
90
100
0
20
40
60
80
100
120

Fig. 6.2


% distance along small intestine
Day Postinfection
Density 6
Density 25
Density 100
0
10
20
30
40
50
60
70
80
90
100
0
10
20
30

At day 16, F=10.85 P<0.001 (D.F=2,39), at day 20, F=104.41 P<0.001 (D.F.=2,41) and at day 24, F=14.34 P<0.001 (D.F.=2,65). These differences though (see Fig. 6.2) are not related in any consistent fashion with exposure density history, so there appears to be no indication from this investigation that mean parasite location in the gut is influenced by parasite density over the density range studied here.

At each of the various metacercarial cyst exposure densities, worms were found to inhabit only the small intestine. The spatial distribution of *E. liei* (see Table 6.3) at each density appears to reveal an anterior population movement from 8 days postinfection onwards and shows that even as the inital metacercarial cyst exposure density increases the worms occupy the same region of the small intestine at successive days postinfection. From Table 6.3 the 40%-80% region of the gut is the major region utilized in all the infection densities from 12 days onwards. At 8 days postinfection the 80%-100% region of the small intestine is utilized by all the infection densities.

6.3.3 Length and width increases

The growth of *E. liei* after varying metacercarial cyst exposure densities using length and width as criteria at each time interval are shown in Table 6.4. Growth patterns after each initial metacercarial cyst density are plotted for length in Fig 6.3 and for width in Fig.6.4 with values expressed as means +/- standard errors. Application of the single factor analysis of variance testing reveals no significant difference in length between each of the exposure metacercarial cyst densities at

day 20, F=2.96 P>0.05 (D.F.=2,13), but a highly significant difference in length at day 16, F=6.66 P<0.01 (D.F.=2,22) and day 24, F=134.57 P<0.001(D.F.=2,36). When applied to width a highly significant difference was observed at day 16, F=17.44 P<0.001 (D.F.=2,22), at day 20, F=14.83 P<0.001 (D.F.=2,13) and day 24, F=267.78 P<0.001 (D.F.=2,36). Although there appear to be significant differences in body measurements on successive days postinfection after each of the initial exposure metacercarial cyst densities, the varying length and width measurements of the worms recovered from each of the initial exposure densities at successive time intervals do not show a pattern that could be attributed to a consistent density effect.

6.3.4 *In-utero* egg counts

Mean *in-utero* egg counts in worms derived from different density exposures and at different times postinfection are summarized in Table 6.5 and described graphically in Fig 6.5. Examination of these results reveal that the metacercarial cyst exposure density 6 infection protocol provided worms which contained a greater number of eggs in their uteri at each successive time interval apartfrom the metacercarial cyst exposure density 25 infection at 20 days postinfection. At 16 days postinfection a plateau at about 800 eggs is reached with regards to the number of *in-utero* eggs in the metacercarial cyst exposure density 6 infection. At day 8 the mean uterine egg counts revealed values of 10, 7.67, and 1 for metacercarial cyst exposure densities 6, 25, and 100 respectively while at day 24 these respective uterine egg counts were 781.5, 659 and 168. As a plateau was reached at day 16, single factor analysis

testing was applied from 16 days onwards to test if there was a significant difference between the uterine egg values at each of the initial metacercarial cyst exposure densities on successive days postinfection. The egg counts were first transformed by a logarithmic transformation as recommended by Elliot (1983). Significant differences were revealed at day 16 where $F=6.30$ $P<0.05$ (D.F.=2,9), and a very highly significant difference revealed at days 20, $F=92.08$ $P<0.001$ (D.F.=2,12) and at day 24, $F=265.07$ $P<0.001$ (D.F.=2,10). Inspection of Fig. 6.5, particularly the curve relating to the 100 cyst exposure density infections, strongly suggests that high density is associated with a reduction in *in-utero* egg counts through time. The multiple worm infections provided sufficient replicated material to examine this relationship more closely. Fig. 6.6 shows the absolute in-uterine egg value number plotted against the actual individual worm intensities per host and reveals a significantly declining pattern of *in-utero* egg counts with increasing infection intensity ($N=81$; $R=0.34$, $P<0.001$).

TABLE 6.4 The mean length and width of worms recovered from the small intestine at various cyst exposure densities

Days postinfection	LENGTH (mm)			WIDTH (mm)		
	Density 6	Density 25	Density 100	Density 6	Density 25	Density 100
8	3.53 (+/-0.05)* n=4	3.96 (+/-0.12) n=8	3.49 (+/-0.07) n=15	0.84 (+/- 0.00)	0.84 (+/-0.02)	0.65 (+/-0.01)
12	5.92 (+/-0.38) n=3	6.10 (+/-0.07) n=7	6.18 (+/-0.07) n=17	1.44 (+/-0.00)	1.31 +/-0.02)	1.09 (+/-0.01)
16	7.30 (+/-0.1) n=2	6.55 (+/-0.12) n=10	6.89 (+/-0.06) n=13	1.52 (+/-0.00)	1.30 (+/-0.03)	1.52 (+/-0.03)
20	7.32 (+/-0.12) n=2	7.13 (+/-0.08) n=4	6.88 (+/-0.09) n=10	1.58 (+/-0.10)	1.70 (+/-0.04)	1.28 (+/-0.05)
24	7.82 (+/-0.02) n=2	9.37 (+/-0.11) n=9	6.75 (+/-0.07) n=28	1.68 (+/-0.00)	1.94 (+/-0.04)	1.19 (+/-0.01)

* Figures in brackets refer to the standard error of the mean length and width values
n values for mean width as for mean length

Fig. 6.3 Mean length at various cyst exposure densities
(data points include standard errors)

Fig. 6.4 Mean width at various cyst exposure densities
(data points include standard errors)

Fig 6.3

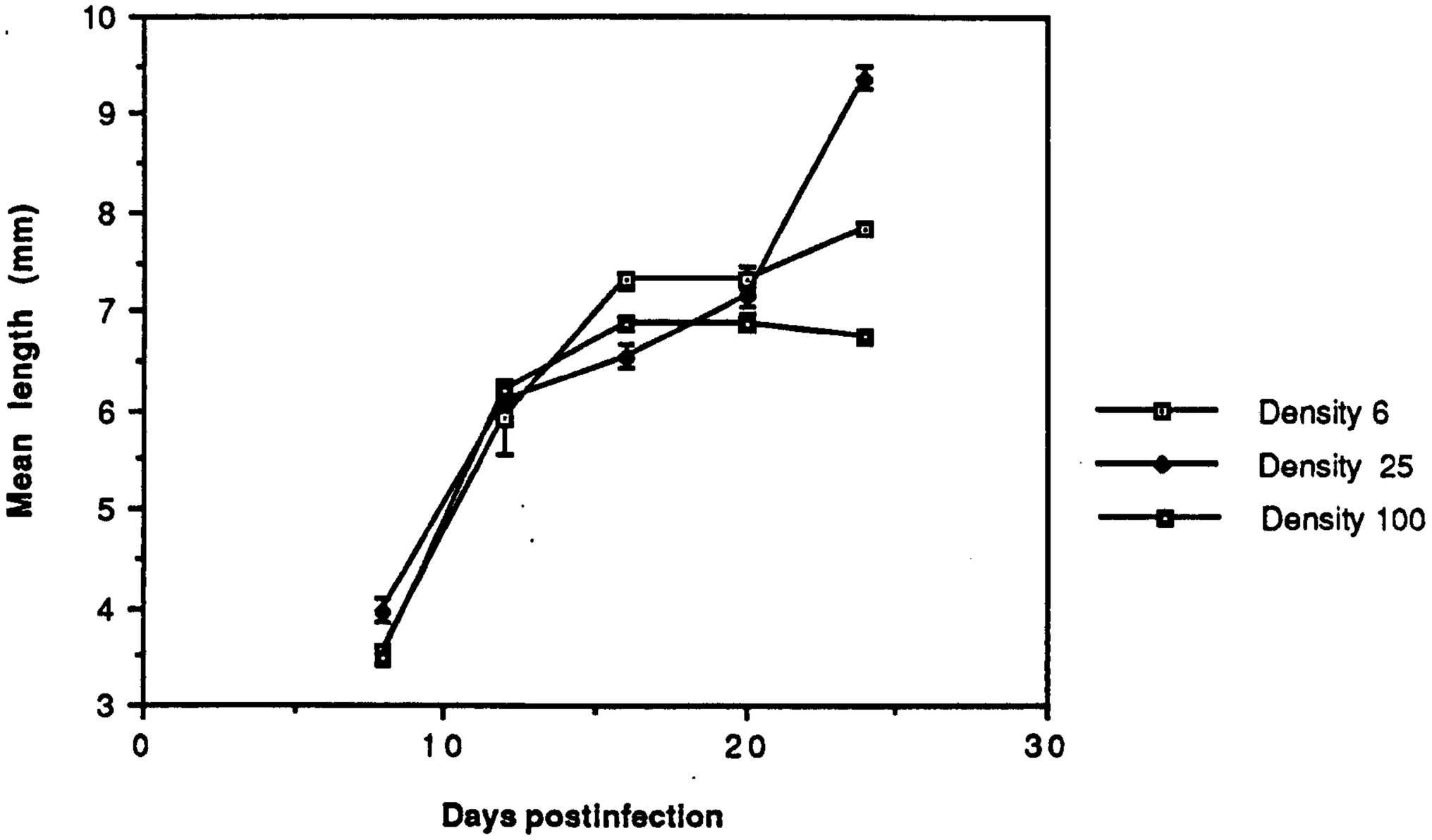

Mean length (mm)
10
9
8
7
6
5
4
3
0
10
20
30
Days postinfection
Density 6
Density 25
Density 100

Fig. 6.4

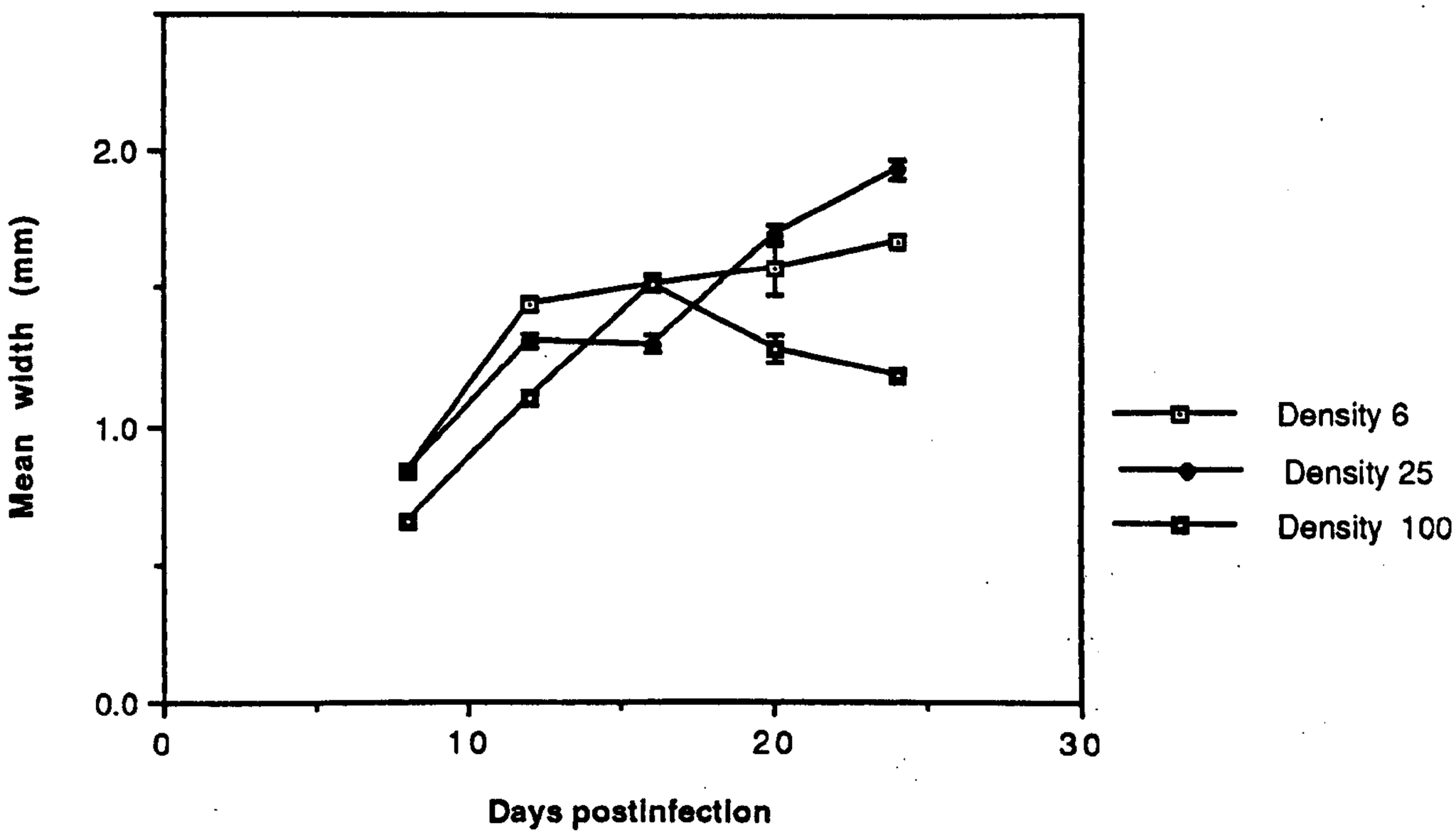

Mean width (mm)
2.0
1.0
0.0
0
10
20
30
Days postinfection
Density 6
Density 25
Density 100

TABLE 6.5 The mean number of eggs recovered in-utero at various cyst exposure densities

Days postinfection	Density 6	Density 25	Density 100
8	10.00 (+/-2.23)* n=5	7.67 (+/-2.83) n=15	1.00 (+/-0.68) n=10
12	405.00 (+/-47.1) n=2	352.70 (+/-7.92) n=3	202.80 (+/-16.00) n=6
16	772.00 (+/-3.000 n=2	455.00 (+/-44.90) n=5	546.40 (+/-31.700 n=5
20	767.50 (+/-10.5) n=2	792.70 (+/-12.59) n=3	283.10 (+/-13.85) n=10
24	781.50 (+/-9.47) n=2	659.00 (+/-31.90) n=4	168.00 (+/-7.84) n=7

* Figures in brackets refer to standard errors of mean values

Fig. 6.5 Mean number of *in-utero* eggs at various cyst exposure densities (data points include standard errors)

Fig. 6.6 Uterine egg numbers at the established worm densities

Fig 6.5

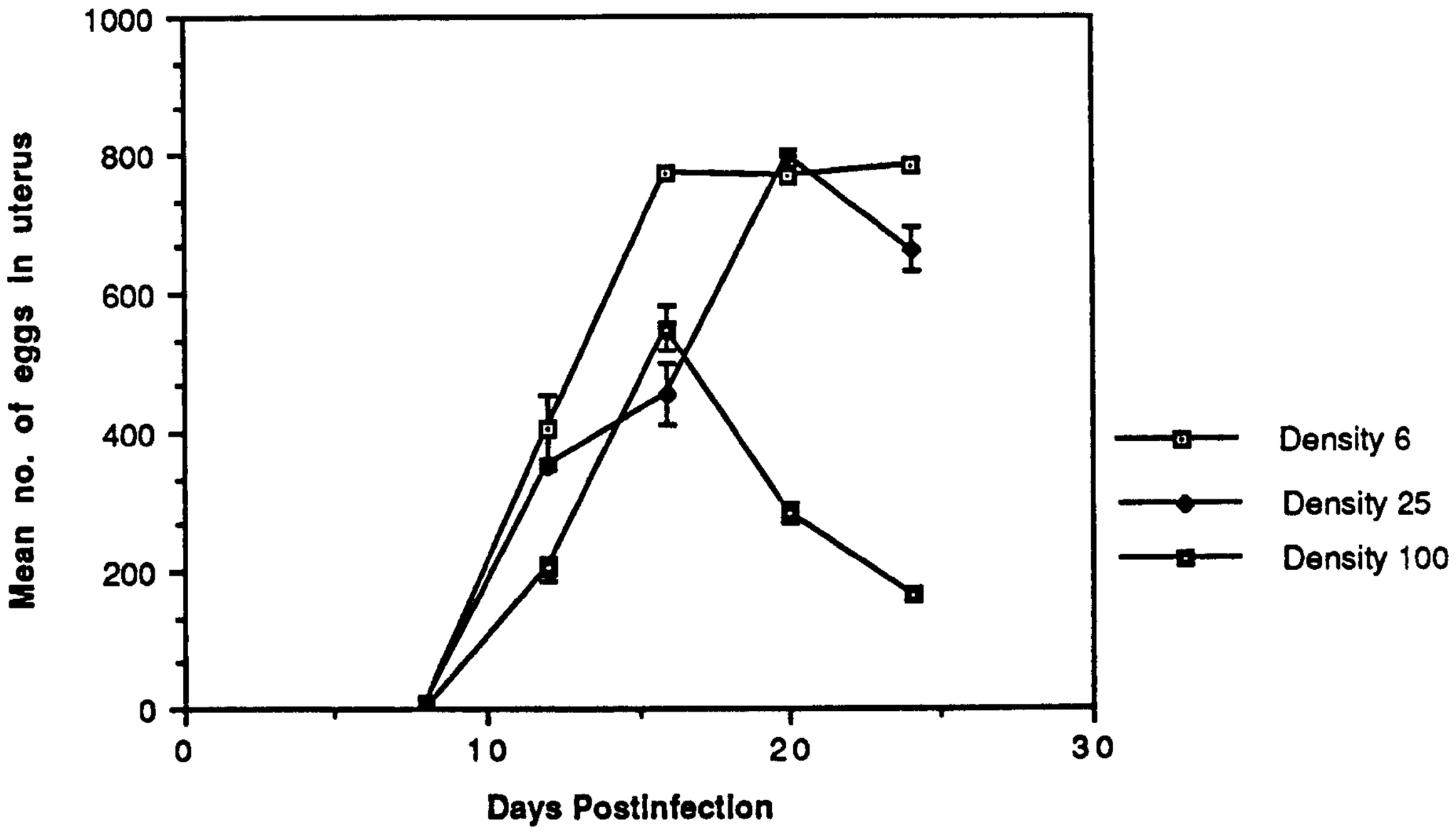
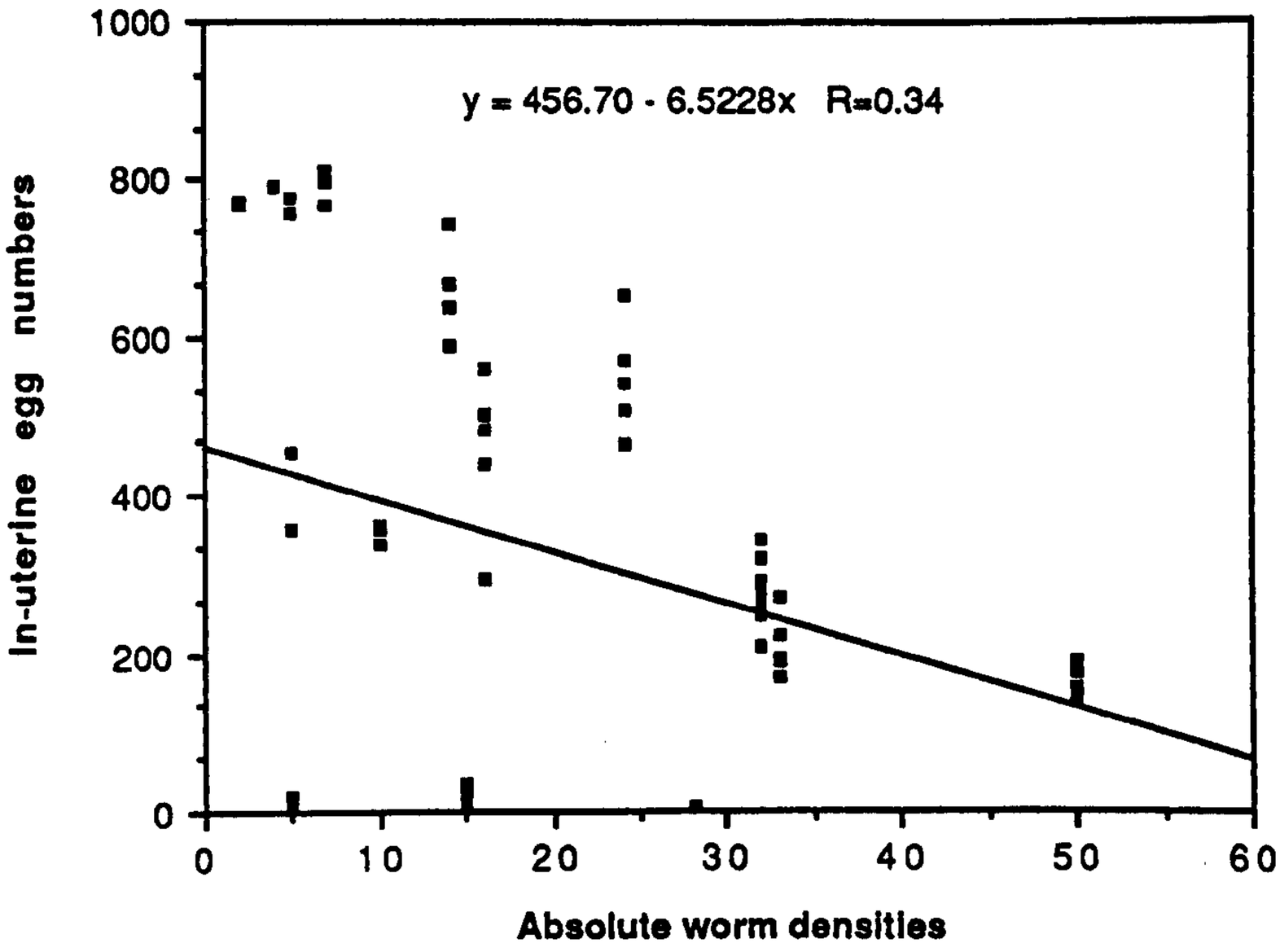

1000
800
600
400
200
0
0
10
20
30
Mean no. of eggs In uterus
Days Postinfection
Density 6
Density 25
Density 100

Fig.6.6


1000
800
600
400
200
0
0
10
20
30
40
50
60
y = 456.70 - 6.5228x R=0.34
In-uterine egg numbers
Absolute worm densities

6.3.5 Total egg ouput over 24 hours

The mean number of eggs deposited in 24 hours, that is the total number of eggs per day (EPD), produced by all the worms at each infection level are summarized in Table 6.6 and represented graphically in Fig.6.7. Analysis of the faecal ouputs of the mice revealed that worms were emitting eggs at day 8 and onwards at every time interval examined in the metacercarial cyst exposure density 6 and 25 infection. No eggs were recovered in the faeces of the metacercarial cyst exposure density 100 infection at 8 days postinfection. Thereafter at this infection intensity, eggs were recovered from the faecal preparations at each time interval. The egg output by the worms at each time period in the metacercarial cyst exposure density 6 infection appeared to increase uniformly, the mean egg output in 24 hours rising from 83.4 at day 8 to 9481.2 at 24 days postinfection. This uniform pattern is in complete contrast to that exhibited by both the metacercarial cyst exposure density 25 and 100 infections. At the exposure density 25 infection the mean egg output over 24 hours rises from 368.8 at day 8 to 15514.7 at day 16 but then decreases to 11167.1 at 24 days postinfection. A similar but more extreme pattern is seen at

TABLE 6.6 The number of eggs expelled per day (EPD) by E.liei at various cyst exposure densities

Days postinfection	Density 6	Density 25	Density 100
8	83.45 (+/-12.85)*	368.80 (+/-40.94)	0.00 (+/-0.00)
12	5592.30 (+/-508.80)	9363.70 (+/-352.30)	10771.40 (+/-1419.60)
16	6343.70 (+/-334.60)	15514.70 (+/-1195.40)	33645.50 (+/-6454.50)
20	9349.00 (+/-603.80)	8294.80 (+/-303.60)	13640.10 (+/-489.00)
24	9481.20 (+/-489.30)	11167.10 (+/-725.60)	8922.80 (+/-583.30)

* n= 2 for each value. Figures in brackets denote the standard error of the mean EPD.

TABLE 6.7 The mean number of eggs per worm per day at various cyst exposure densities

Days postinfection	Density 6	Density 25	Density 100
8	16.69 (+/-2.57)*	24.59 (+/-2.73)	0.00 (+/-0.00)
12	1118.5 (+/-101.7)	936.40 (+/- 35.21)	326.40 (+/-42.99)
16	3171.80 (+/-167.30)	969.60 (+/-74.74)	1401.90 (+/-268.90
20	1869.8 (+/-120.80)	1184.90 (+/-43.34)	426.20 (+/-15.20)
24	2370.30 (+/-122.50)	797.60 (+/-51.83)	178.40 (+/-10.75)

*Figures in brackets refer to standard errors

TABLE 6.8 The number of eggs per gram of faeces in faecal samples recovered from mice infected with various cyst exposure densities.

Days postinfection	Density 6	Density 25	Density 100
8	23.65 (+/-3.65)*	115.6 (+/-12.8)	0.00 (+/-0.00)
12	2519 (+/-229.2)	2256.3 (+/-84.9)	5228.80 (+/-689.1)
16	1762.1 (+/-92.9)	4181.8 (322.2)	10195.6 (+/-1955.8)
20	2314.10 (+/-149.5)	2126.9 (+/-77.8)	3247.60 (+/-161.4)
24	1950.9 (+/-139.6)	3084.8 (+/-200.5)	2241.9 (+/-187.4)

n=2 for each value * Figures in brackets refer to standard errors

Fig. 6.7 Total egg output in 24 hours (EPD) (data points include standard errors)

Fig. 6.8 Total number of eggs per worm in 24 hours (data points include standard errors)

Fig.6.7

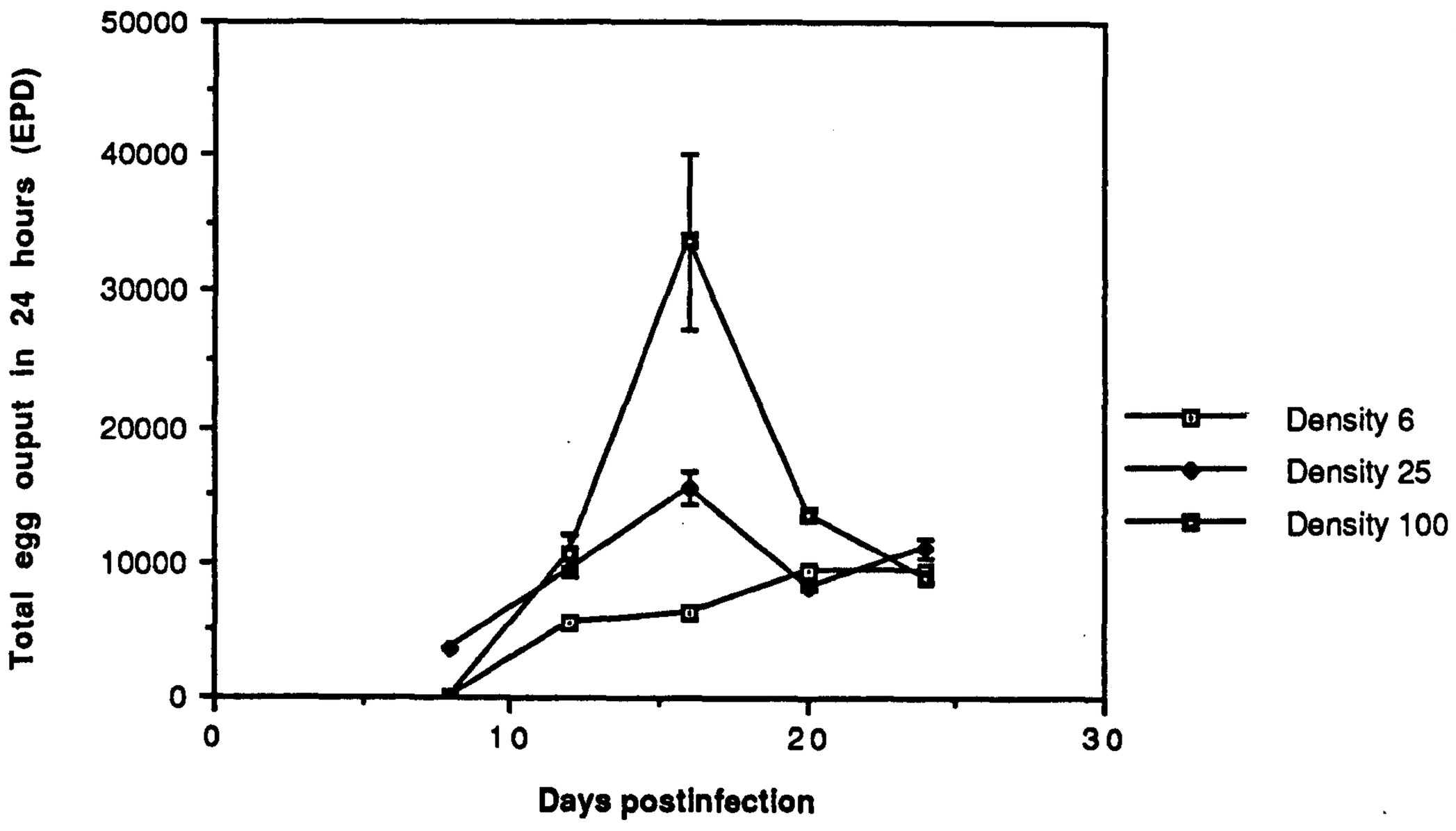
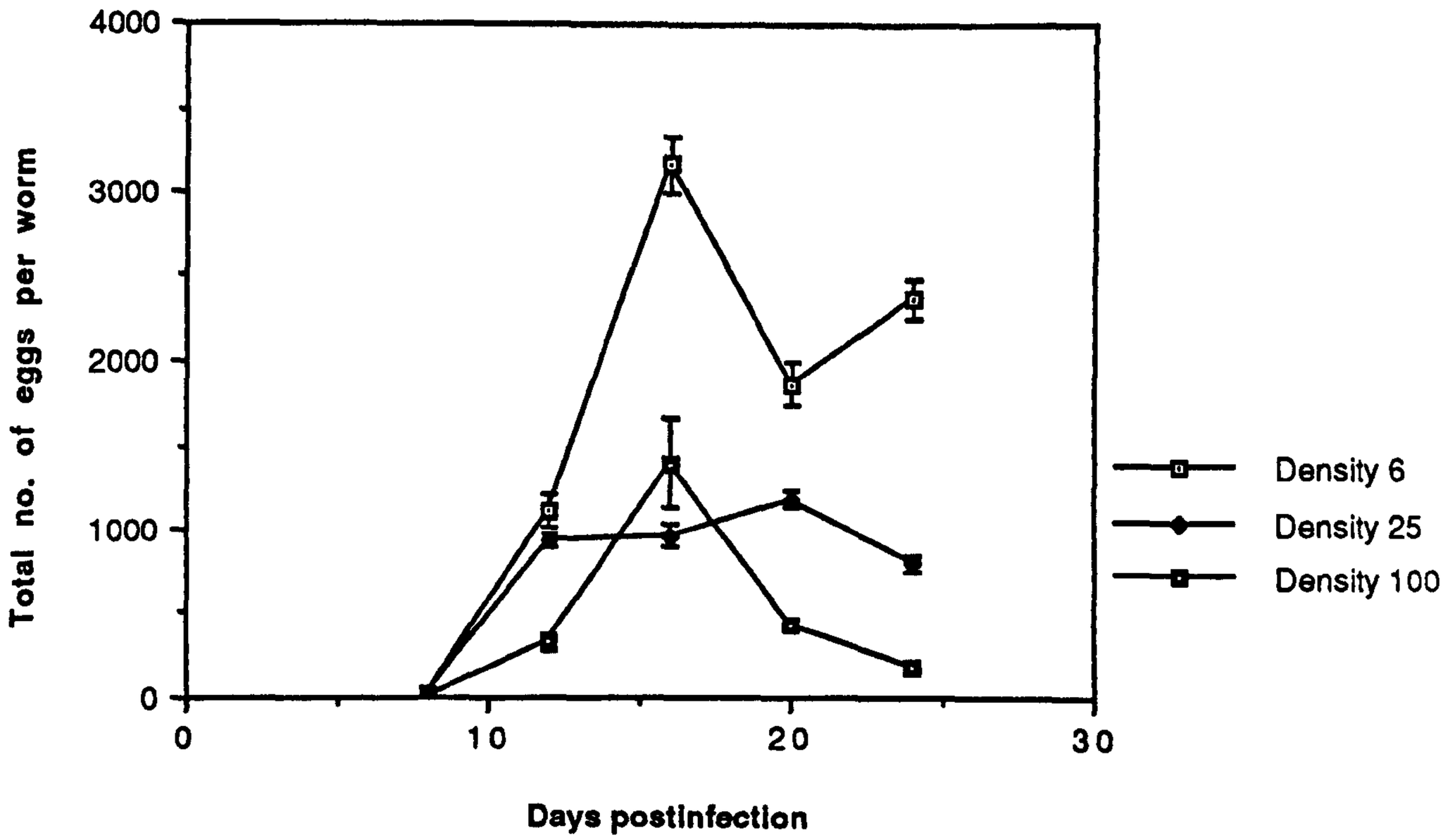

50000
40000
30000
20000
10000
0
Total egg ouput in 24 hours (EPD)
0
10
20
30
Days postinfection
Density 6
Density 25
Density 100

Fig.6.8


4000
3000
2000
1000
0
Total no. of eggs per worm
0
10
20
30
Days postinfection
Density 6
Density 25
Density 100

the metacercarial cyst exposure density 100 infection with mean egg ouput rising to 33645.5 on day 16 falling to 8922.8 on day 24.

6.3.6 Egg output per worm in 24 hours

Density-dependent constraints appear to have an effect on the number of eggs released in 24 hours by each worm at each infection level, resulting in lower numbers of eggs being released per worm as the infection density increases. The mean number of eggs released in 24 hours per worm at each density over successive days postinfection are presented in Table 6.7 and displayed graphically in Fig 6.8. Fig 6.8 shows that the egg production capacity of each worm in the metacercarial cyst exposure densty 6 infection from day 12 to day 24 is far greater than each corresponding value in the 25 and 100 intensity infection. Single factor analysis of variance testing, (with the number of eggs per worm transformed logarithmically) was used to determine if there was any significant difference between the mean egg output per worm values after each of the initial metacercarial cyst exposure densities on successive days postinfection. Significant differences were revealed at day 16, $F=23.96$ $P<0.05$ (D.F.=2,3), at day 20, $F=252.32$ $P<0.001$ (D.F.=2,3) and day 24, $F=479.99$ $P<0.001$ (D.F.=2,3).

To examine this relationship further the total number of eggs produced per worm in the whole 24 days of the experiment after each of the exposure densities was estimated. The estimation technique contained the assumptions that egg output rates were essentially continuous once they had begun and that

between the sampled days in the sequence, rates altered smoothly and linearly. This estimation provided the global total egg production per average worm up to 24 days postinfection. These values for the initial metacercarial cyst exposure densities of 6, 25 and 100 were estimated as 30,567.09, 14,368.09 and 8,677.9 respectively. The cumulative number of eggs per worm over the 24 days of the experiment is represented graphically in Fig 6.9. This graph and the global number of eggs per worm produced up to 24 days show clearly that density-dependent constraints are operating on the egg producing capability of the worms.

To examine the relationship between the mean number of eggs expelled per worm in 24 hours and the number of *in utero* eggs present at the same time, two types of analysis were carried out. First, the relationship between uterine egg number (EPU) and mean egg output per 24 hours per worm ($\bar{x}$EPW) was considered across the whole range of actual worm densities over the days of the experiment. The relationship is described in Fig. 6.10a which reveals a clear linear positive relationship (N=67; R=0.846;P<0.001) between these two variables.

Fig.6.9 Estimated cumulative egg output per worm at various cyst exposure densities

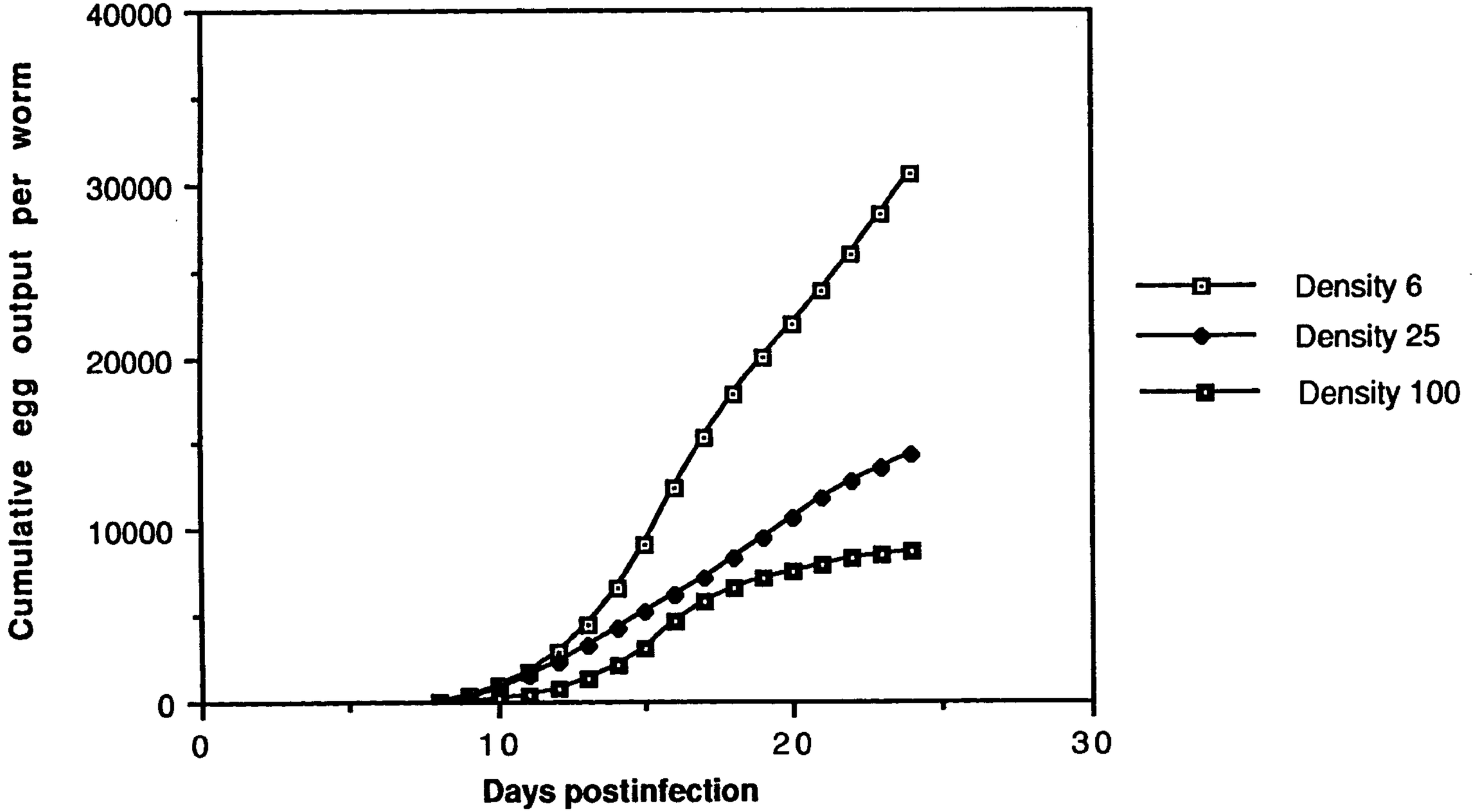


Fig. 6.10a The relationship between mean egg output per worm in 24 hours and uterine egg number

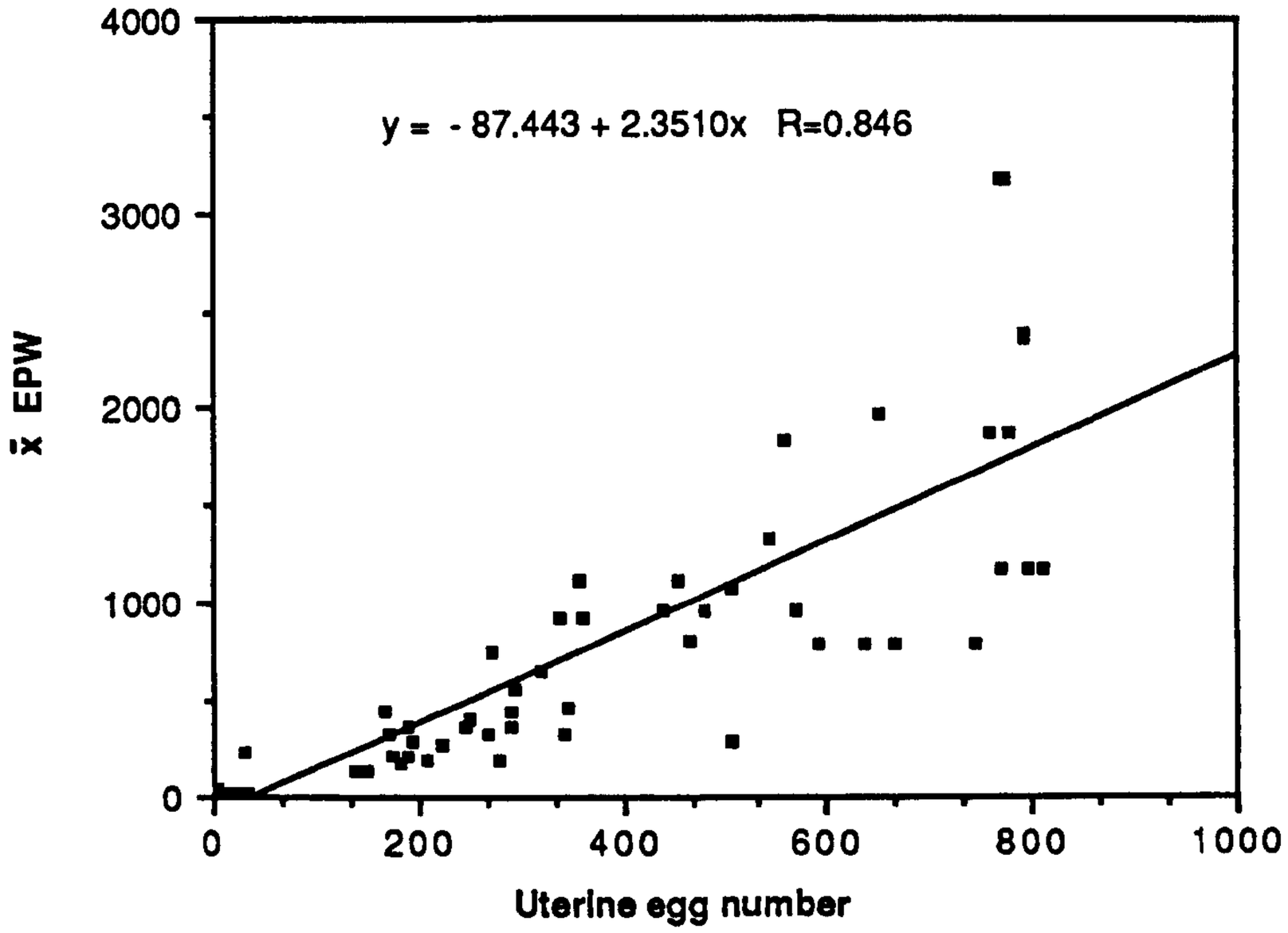


Fig.6.10b The relationship between x̄EPW/EPU and worm density

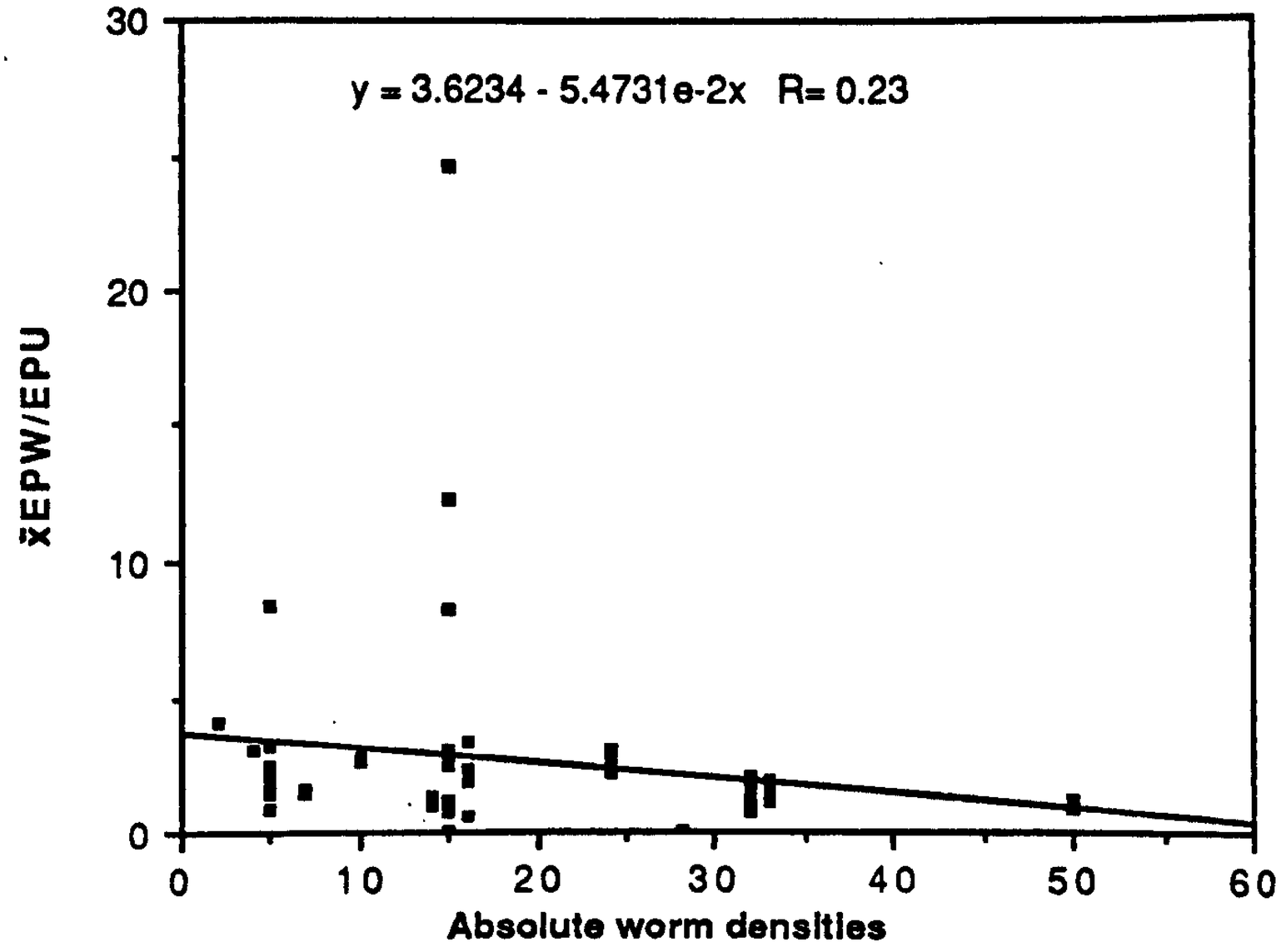


Fig. 6.11

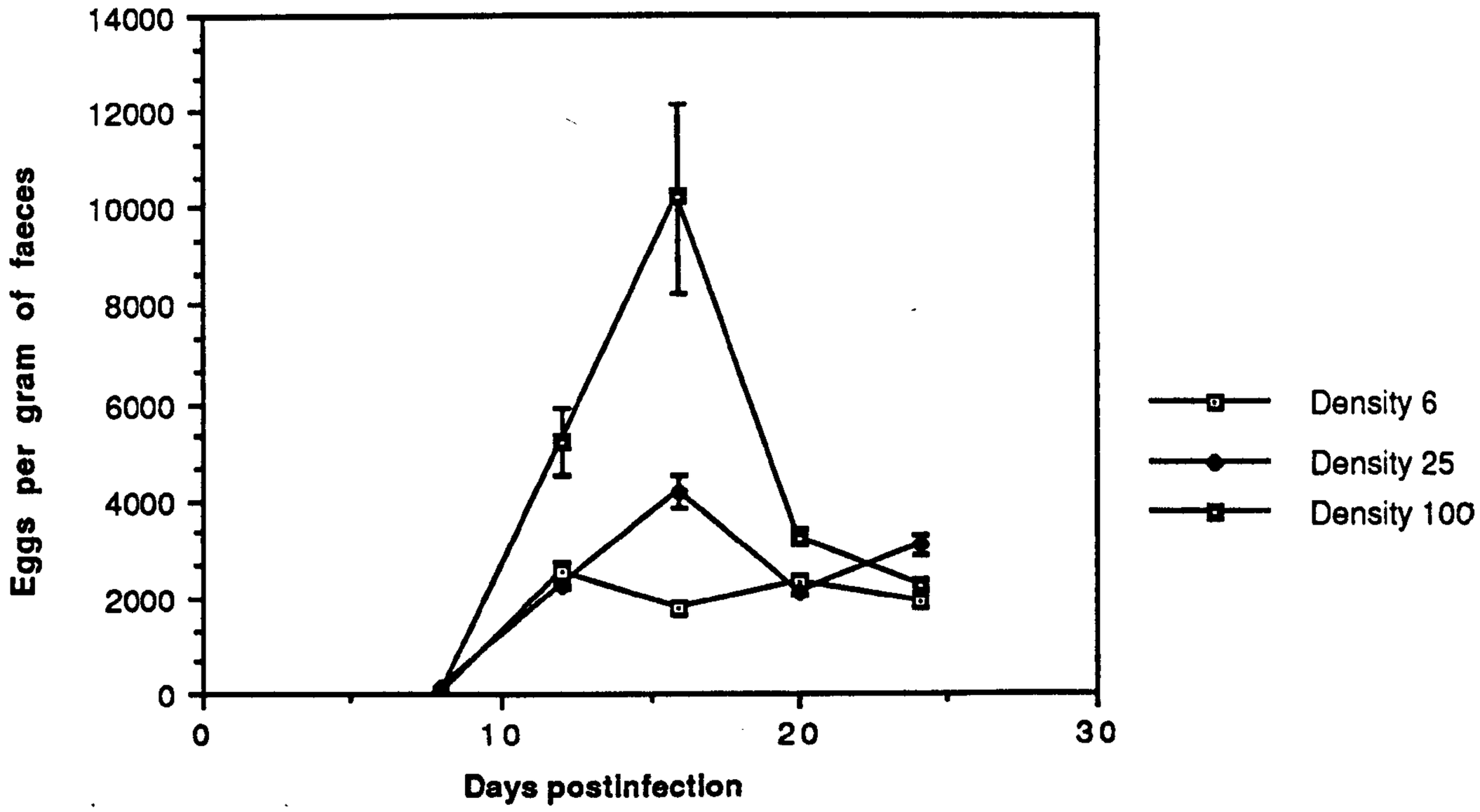


Fig.6.11 Total number of eggs per gram of faeces (EPG) (data points include standard errors)

Second, the ratio of egg output per 24 hours per worm and uterine egg number ($\bar{x}$EPW/EPU) was plotted against absolute worm densities (see Fig. 6.10b). This analysis showed that there was no significant trend in the value of the ratio with increasing worm density (N=67; R=0.23; P>0.05).

6.3.7 Eggs per gram of faeces

In section 6.3.6 cumulative egg numbers over the 24 days of the experiment for each of the initial metacercarial cyst densities confirmed the presence of density-dependent constraints on egg production. In many previous studies on helminth egg production, this process has been measured by reference to egg output as eggs per gram of faeces (EPG). To enable comparisons with other studies egg output in the present system has also been examined in this way. These mean EPG values are shown in Table 6.8 and Fig 6.11 which expresses graphically the mean EPG with respect to successive days postinfection. The pattern of output revealed are very similar indeed to those obtained previously for total egg output per infection per 24 hours (EPD) (see Fig. 6.7).

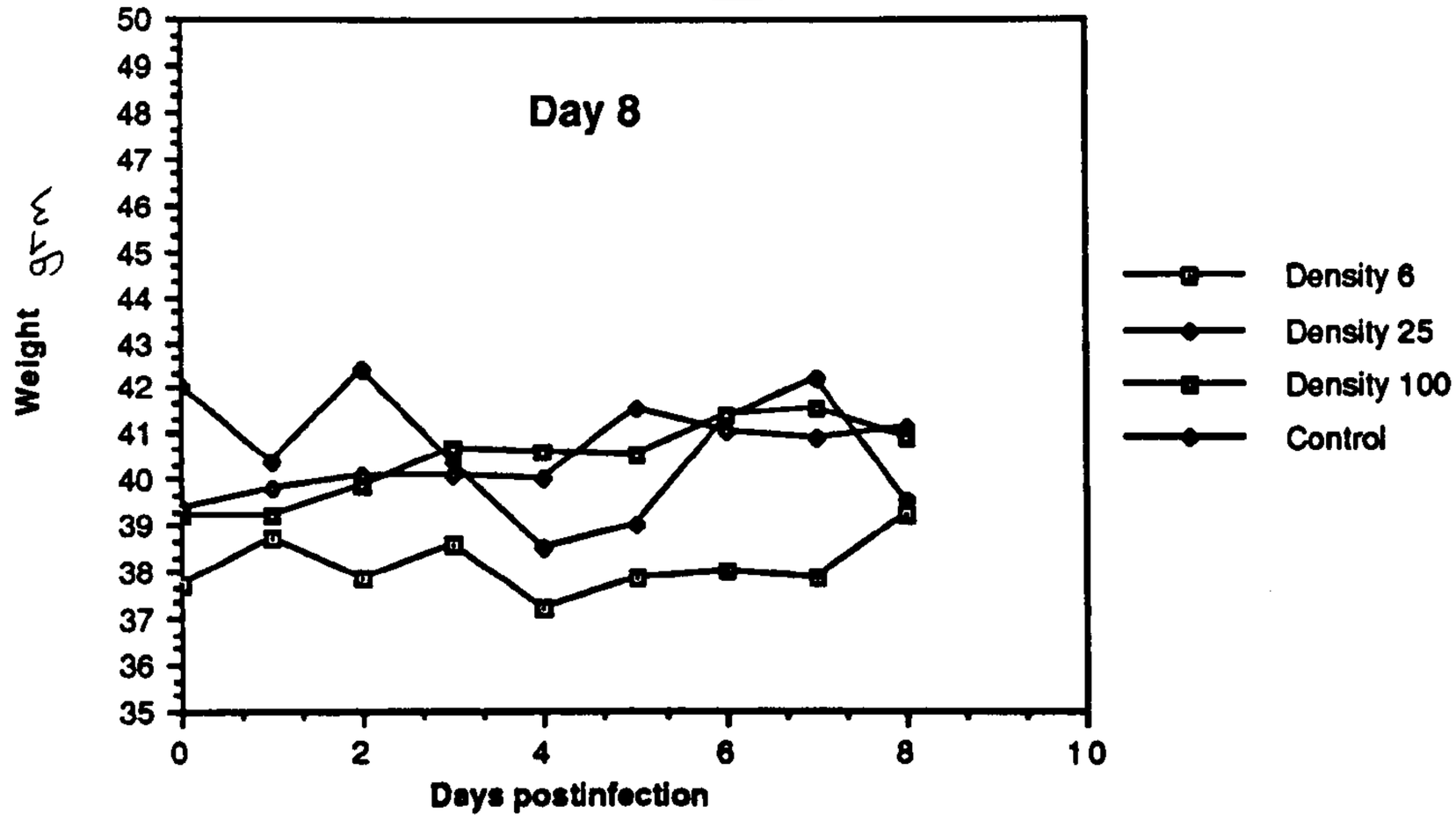
6.3.8 Clinical signs and blood parameters

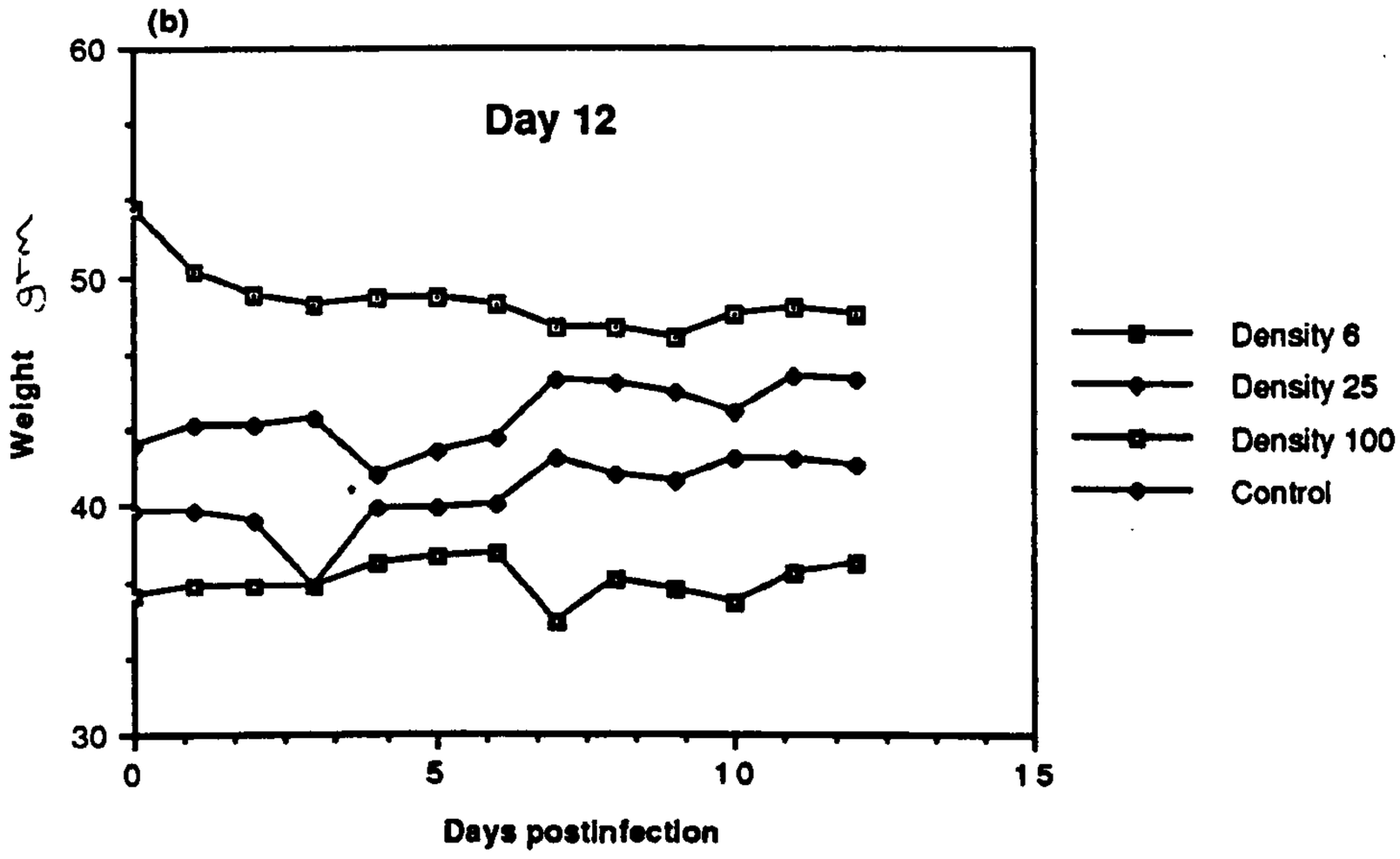
During the course of this 24 day long experiment there were no apparent differences between the external appearance and behaviour of the control and experimental mice. All mice appeared to behave normally, showed no signs of any weakness and their coats appeared well groomed. There appeared to be no dramatic weight lossess or gains at any of the infection levels and the weight changes that were observed were comparable to

those of control mice.These weight changes with respect to time at each metacercarial cyst exposure density are expressed graphically in Fig. 6.12 (a-e).

Infection intensity-related variation appeared to be exhibited to a small degree by the blood parameters. The haematocrit values (PCV) are summarized in Table 6.9 and Fig 6.13 shows the mean PCV for mice infected with various initial metacercarial cyst exposure densities killed at successive days postinfection. Fig 6.13 shows that there are relatively high PCV values in the 25 and 100 metacercarial cyst infections by day 12 postinfection with a value of 45.6 at this day in the exposure density 100 infection and 47.8% in the exposure density 25 infection. This is followed by a dramatic decrease in mean PCV at both infection levels at day 16 falling to 32.8% and 34.5% in the 25 and 100 metacercarial cyst exposure density infections respectively. From these low levels there is a slight recovery at days 20 and 24, amounting to an increase, reaching 39.1% in the density 25 infection and 37.4% in the density 100 infection by day 24. Even so, these mean PCV values are still notably depressed when compared with control mice. The PCV, for

Fig. 6.12 (a) Weight of mice Infected with varying cyst densities of E.liei





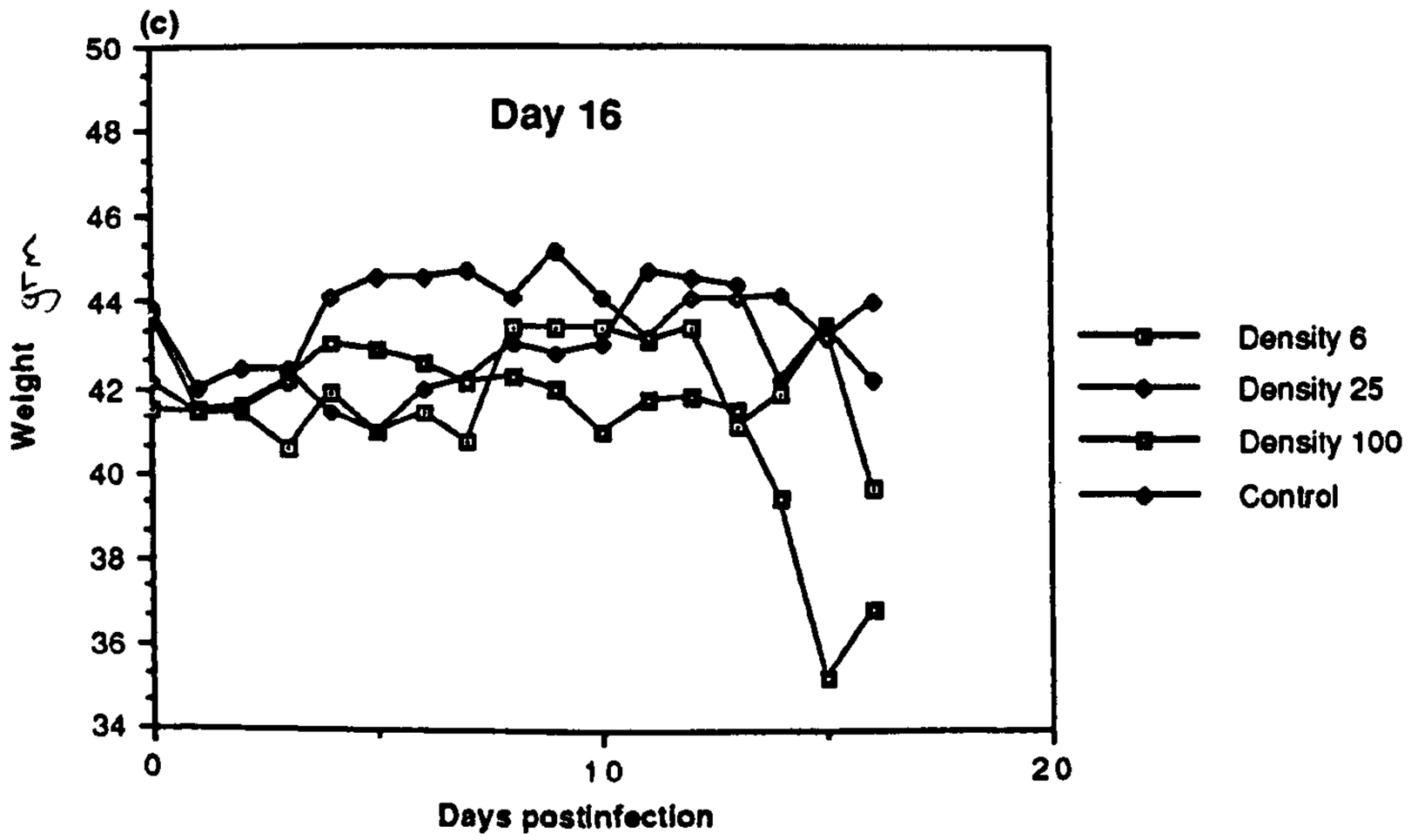
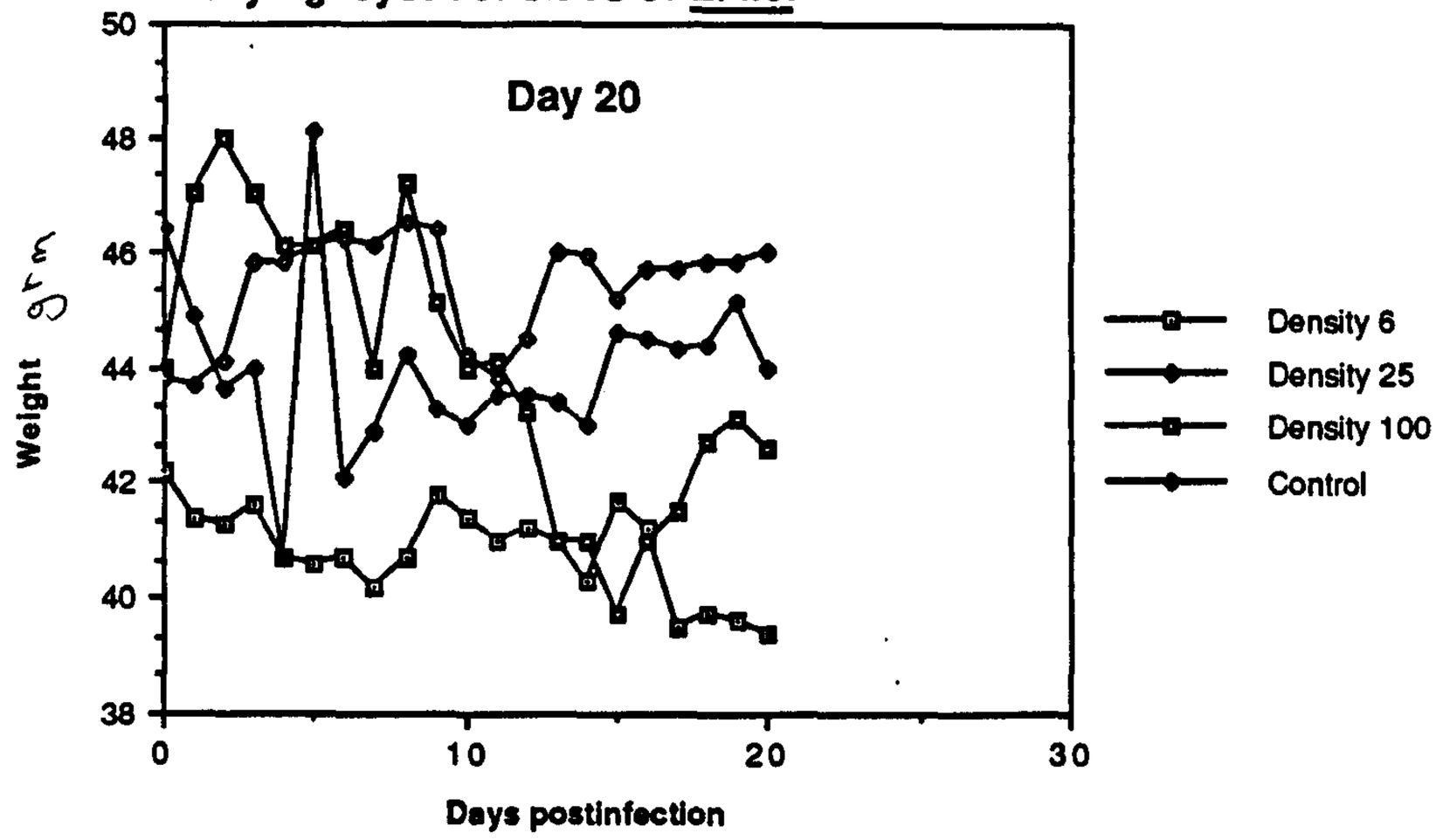


Fig. 6.12 (d) Weight of mice infected with varying cyst densities of E. lieI



(e)

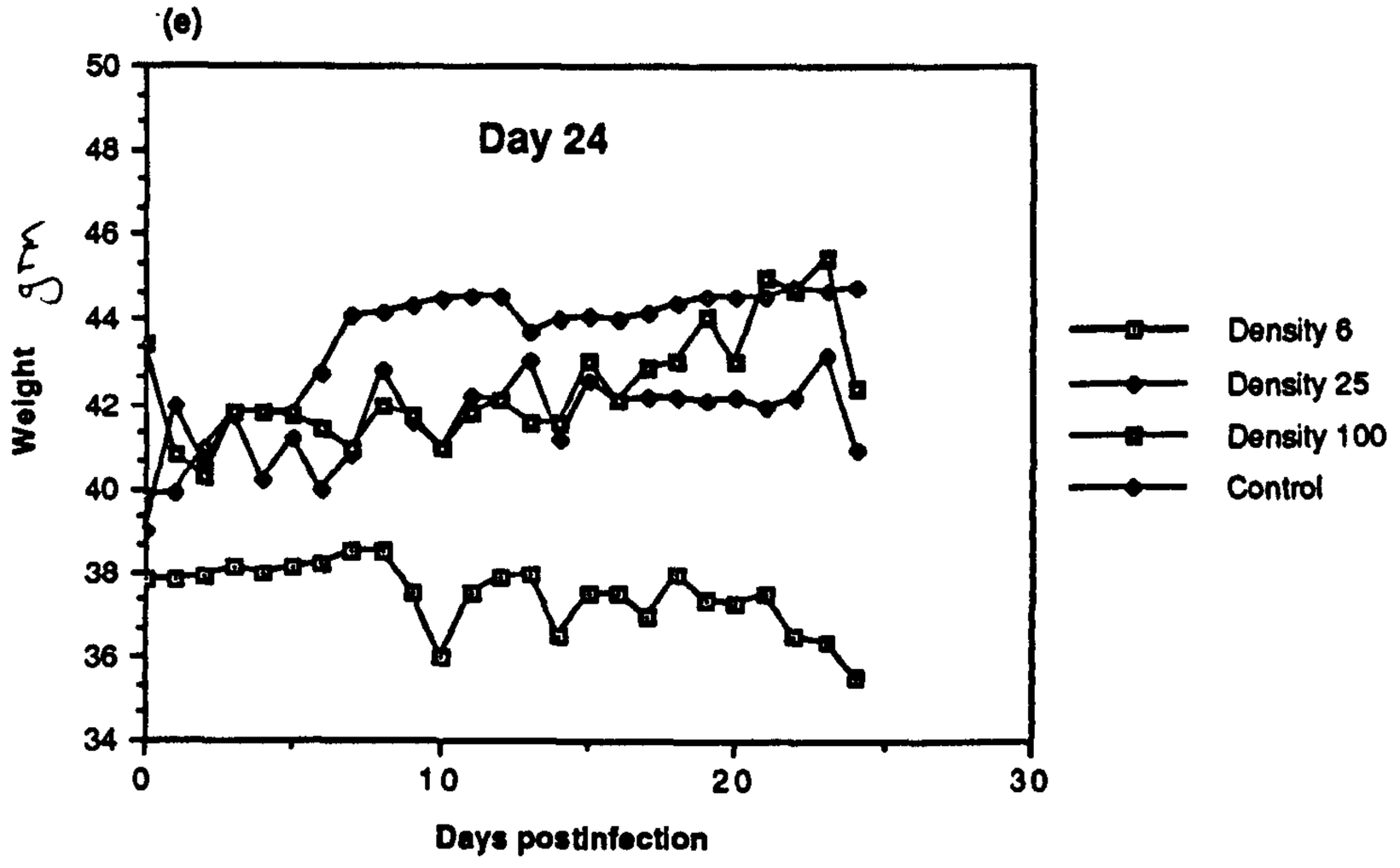


TABLE 6.9 The mean haematocrit values of mice infected with various cyst exposure densities

Days postinfection	Initial cyst density	Mean PCV(%)	Standard error of mean PCV (+/-)
8	6	42.50*	1.50
8	25	41.75	0.25
8	100	49.50	1.50
8	Control	41.60	0.40
12	6	39.10	2.71
12	25	47.80	1.45
12	100	45.60	2.16
12	Control	38.60	0.25
16	6	42.00	0.00
16	25	32.80	0.50
16	100	34.50	0.50
16	Control	40.65	0.45
20	6	40.30	0.30
20	25	36.70	1.91
20	100	36.00	0.00
20	Control	42.00	0.00
24	6	42.00	0.00
24	25	39.10	1.90
24	100	37.40	0.30
24	Control	41.75	0.25

*n=2 for each value

Fig. 6.13 The mean haematocrit values at varying cyst densities

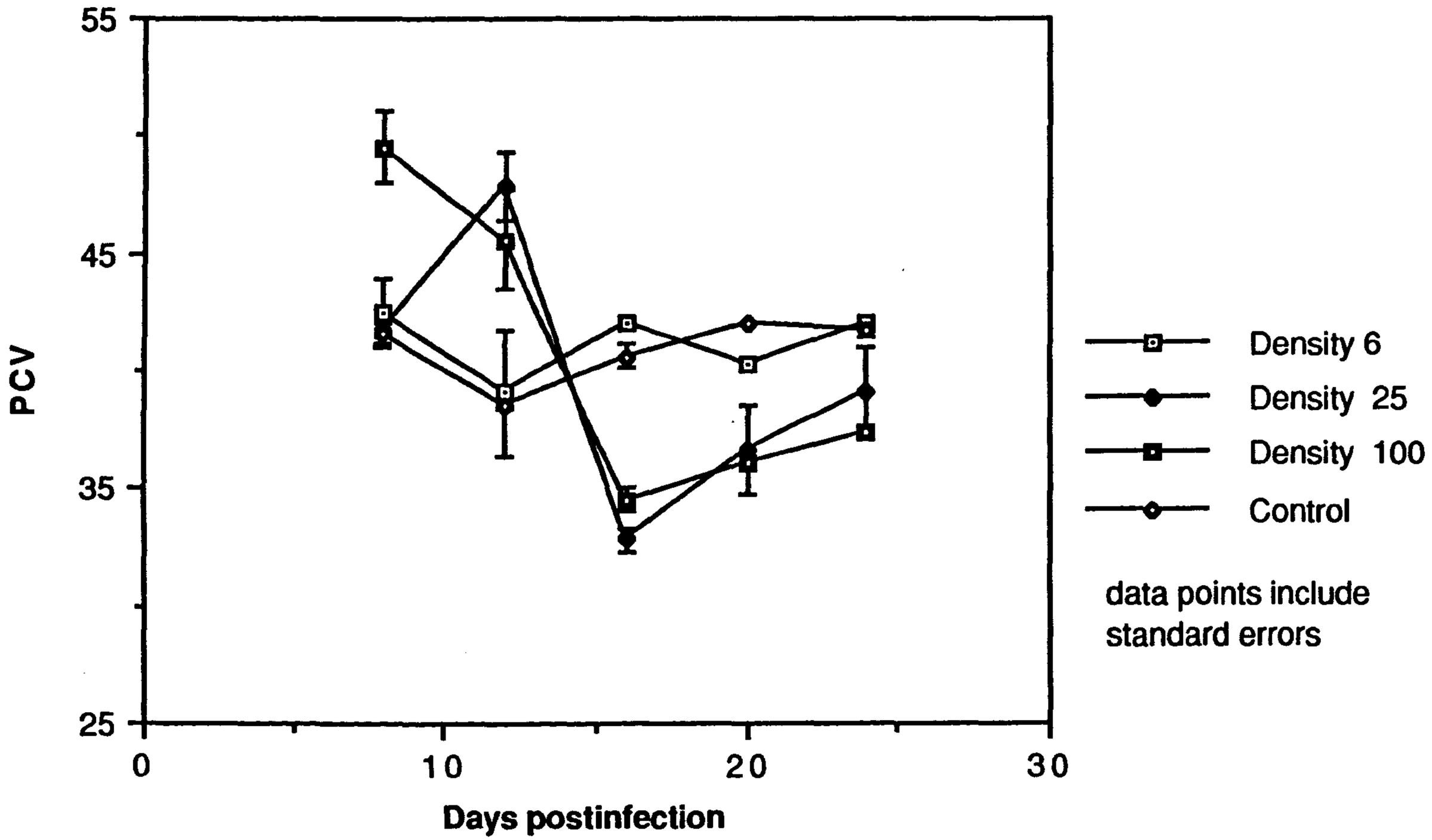


Fig.6.14(a) The differential leucocyte count for mice

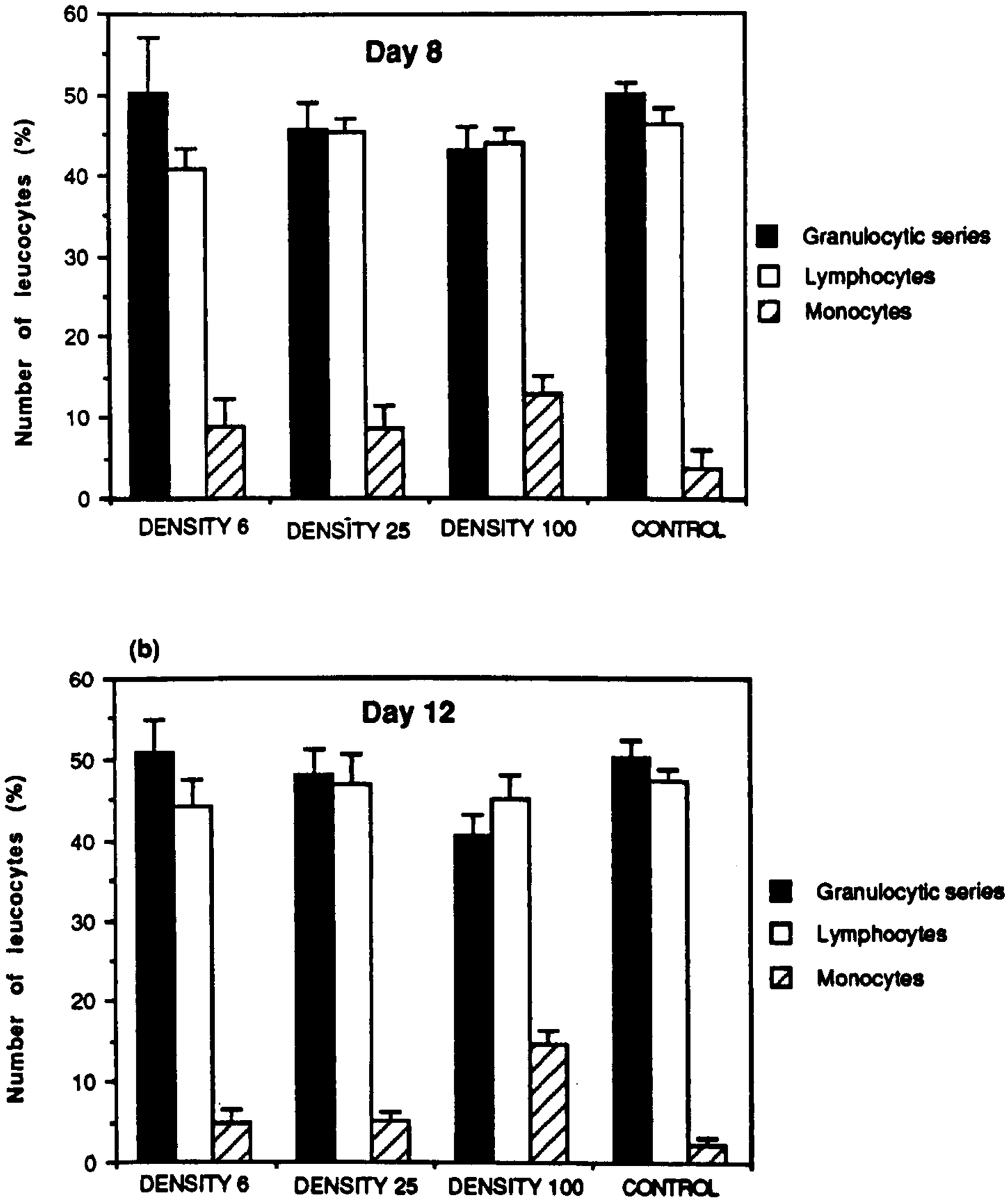
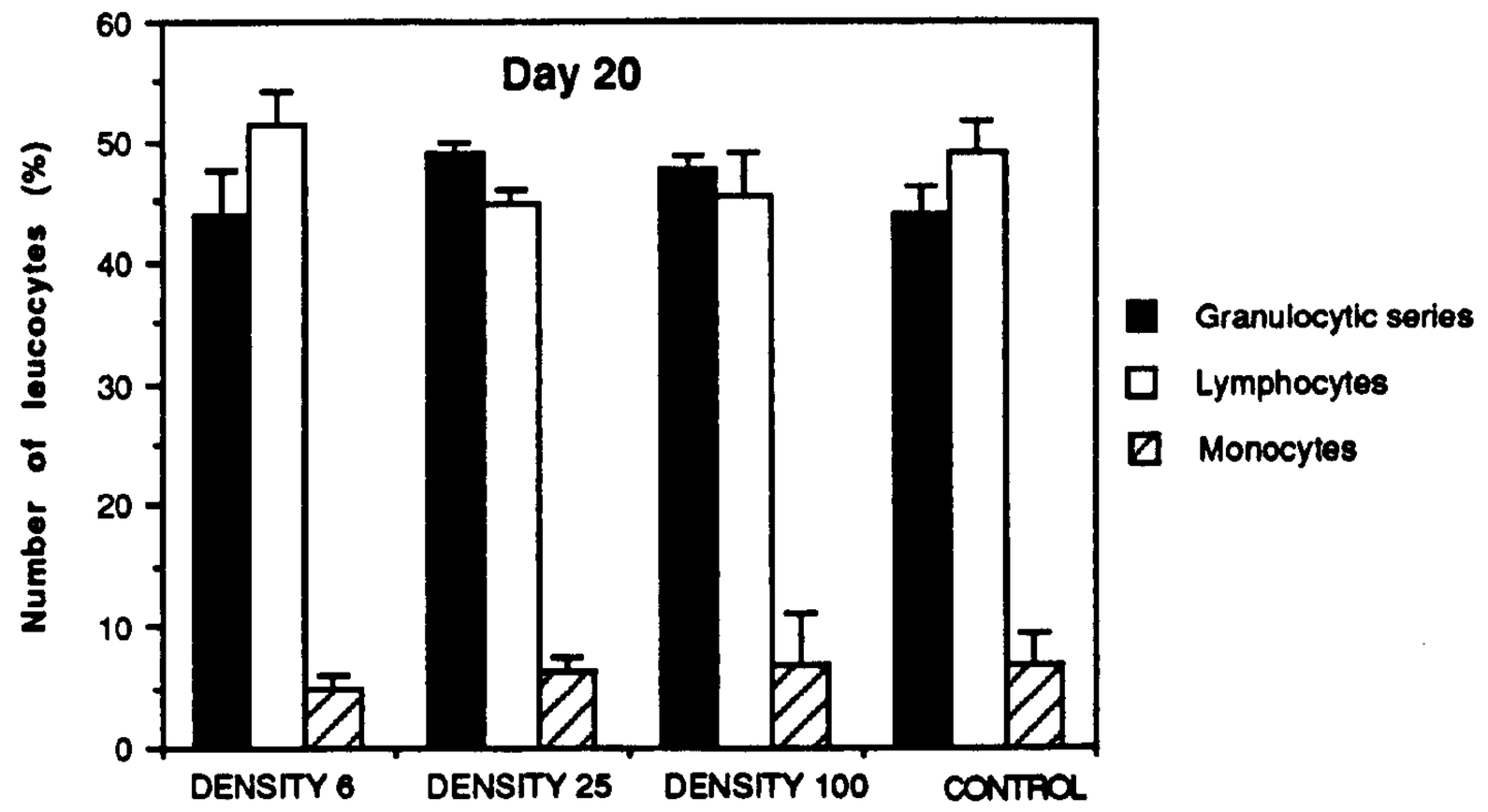
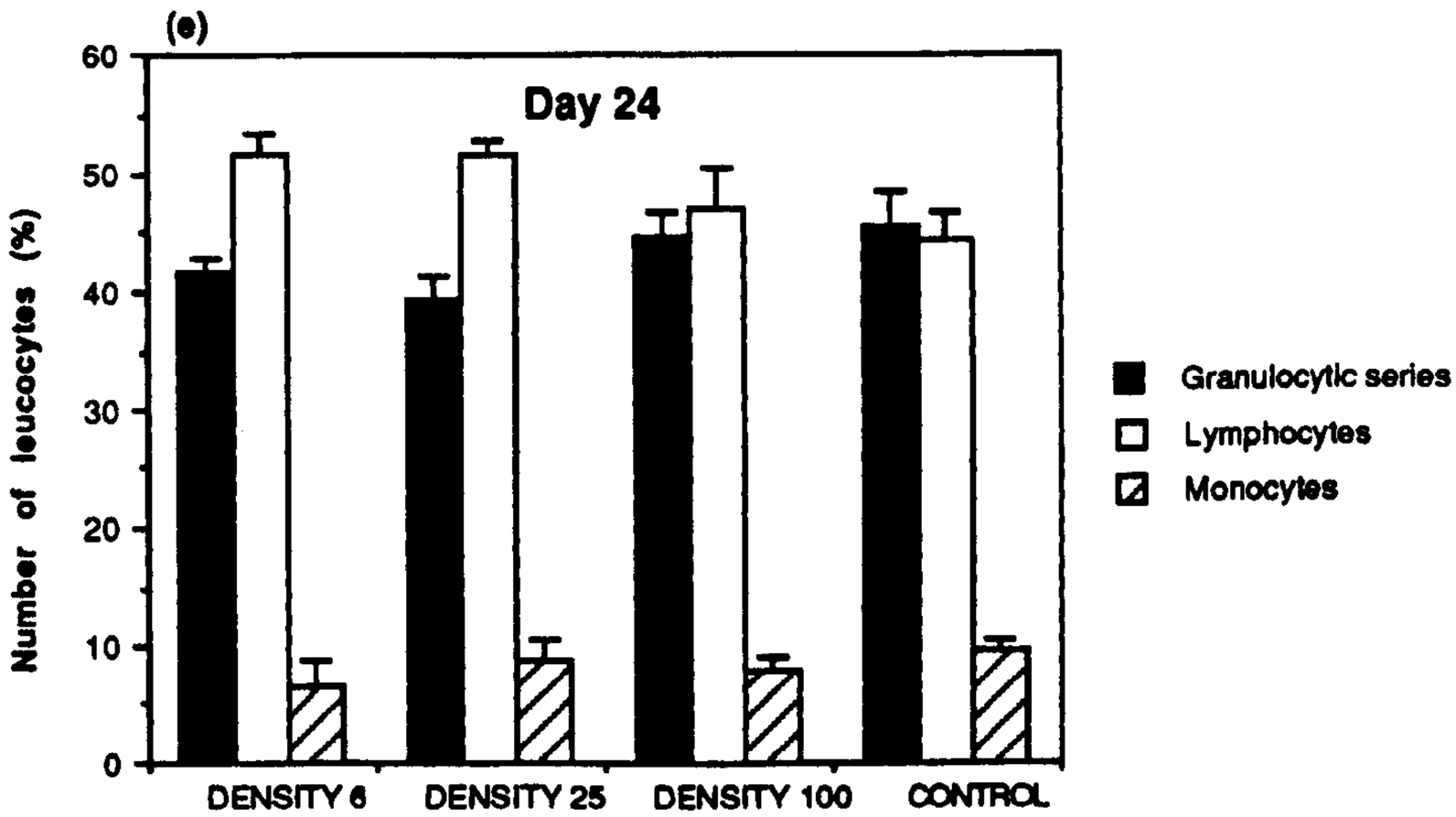


Fig.6.14(d) The differential leucocyte counts for mice





the metacercarial cyst exposure density 6 infection and the controls change very little between days 8 and 24 in contrast to the considerable time-related changes seen at the higher worm density.

No differences were observed regarding the population composition of the leucocytes with respect to the initial metacercarial cyst exposure densities at days 8, 12, 16, 20 and 24. These blood values are summarized graphically in Fig 6.14 (a-e) for each day postinfection. The attached error bars signify the standard error for each mean leucocyte value. Observation of these graphs indicate that there is no marked variation in the components of the leucocytes between the infected individuals and the control mice.

6.3.9 Gross pathology

Marked internal pathological changes were evident in mice harbouring worms in the density range of 24-50 worms per gut that were derived from 100 metacercarial cyst density exposures from 16 days onwards. This pathology involved the ballooning of the infected region of the small intestine which appeared slighty red in colour. These distended regions contained an accumulation of fluid in the gut lumen. Peyer's patches close to infected regions were enlarged when compared with control mice. No marked dilation of the small intestine was observed in the metacercarial cyst exposure density 6 and 25 infections containing numbers of worms in the range of 2-16 per gut over the successive days observed.

6.3.10 Histopathology

Histological examination revealed that in all infected mice pathology was apparent in the intestinal sections which were observed. Areas of the small intestine from uninfected control mice are shown in Fig. 6.15 (1) and (2). Fig 6.15 (1) is a low power view of the jejunum which shows the relationship between the villi (V) and the crypts of Lieberkuhn (C) in an uninfected mouse. The villi, long finger like projections, are prominent and the columnar epithelial cells (E) covering the villi have regularly distributed nuclei. The lamina propria (P) is seen to be arranged in an orderly fashion within each villus and there is a low crypt to villus ratio (approximately 1:4). The tunica muscularis (Tm) is a relatively thin layer of muscle. Fig. 6.15 (2) is a high power view of the tip of a villus in an uninfected mouse showing the internal structure of the villus and the ordered columnar epithelium. The very prominent brush border (B) which outlines the luminal side of the simple columnar epithelium is also clearly apparent.

At all three exposure levels it was revealed that all infected regions had consistent pathological features. These features included crypt hyperplasia with cell and villous atrophy, that is, the flattening and erosion of the villi. Some of the pathological changes with respect to villous atrophy and crypt hyperplasia were moderate in some infections, notably the lower density infections but more severe in others. Fig 6.15 (3) and (4) show an area of the jejunum in the small intestine adjacent to the site of attachment of the worms in a mouse exposed to 6 metacercarial cysts and harbouring 5 worms, at 12 days

postinfection. The shortened, blunt and eroded villi have made the surface of the mucosa appear flat. The orderly structure of the lamina propria has been lost and the nuclei of the simple columnar epithelium have become irregularly distributed. The brush border has been lost completely. There is also an evident desquamation of the epithelial cells of the villi and apparent cellular infiltrations (Ci) into the lamina propria of the eroded villi. This characteristic sculpturing of the intestine was common amongst all the intestines observed at all the metacercarial cyst exposure densities in infected mice.

Fig 6.15 (5) is a region of the jejunum adjacent to the main site of worm attachment in a mouse exposed to 25 metacercariae, harbouring 16 worms at 16 days postinfection. The tips of the villi have been eroded giving rise to the presence of atrophic villi and a flat appearance to the surface of the mucosa. There are hyperplastic crypts and the tunica muscularis has taken on a swollen appearance. A muscular hypertrophy (M) of the tunica

Fig. 6.15 (see Section 6.3.10)

(1) Low power view of the small intestine showing the relationship between the villi and the Crypts of Lieberkuhn in an uninfected mouse.
Scale bar= 25 μm

(2) High power view of villus tip from uninfected mouse
Scale bar= 25 μm

(3) Atrophic villi
Scale bar= 50 μm

(4) High power view of atrophic villi
Scale bar= 25 μm

(5) Atrophic villi with hyperplastic crypts. Note swollen tunica muscularis
Scale bar= 50 μm

(6) Partially eroded villi
Scale bar= 50 μm

Key

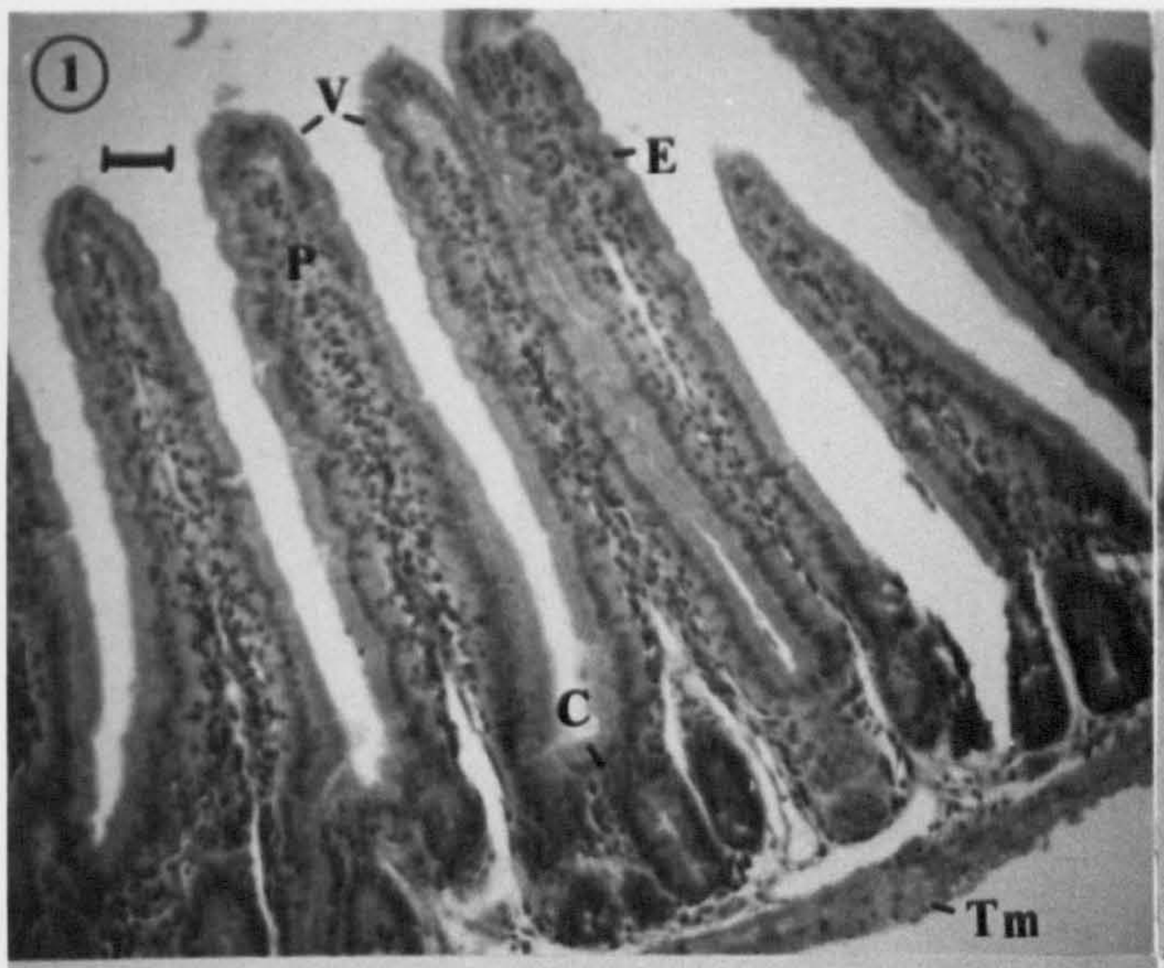
V-villi E-columnar epithelial cells

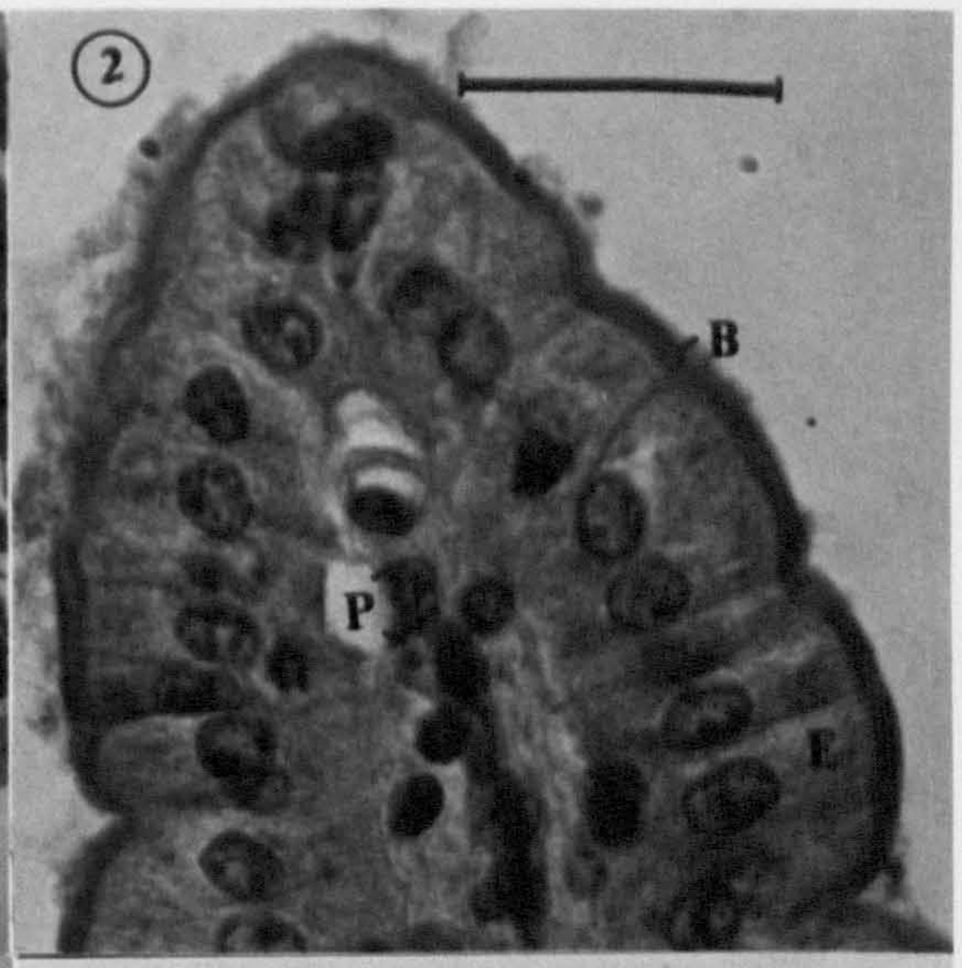
C-Crypts of Lieberkuhn

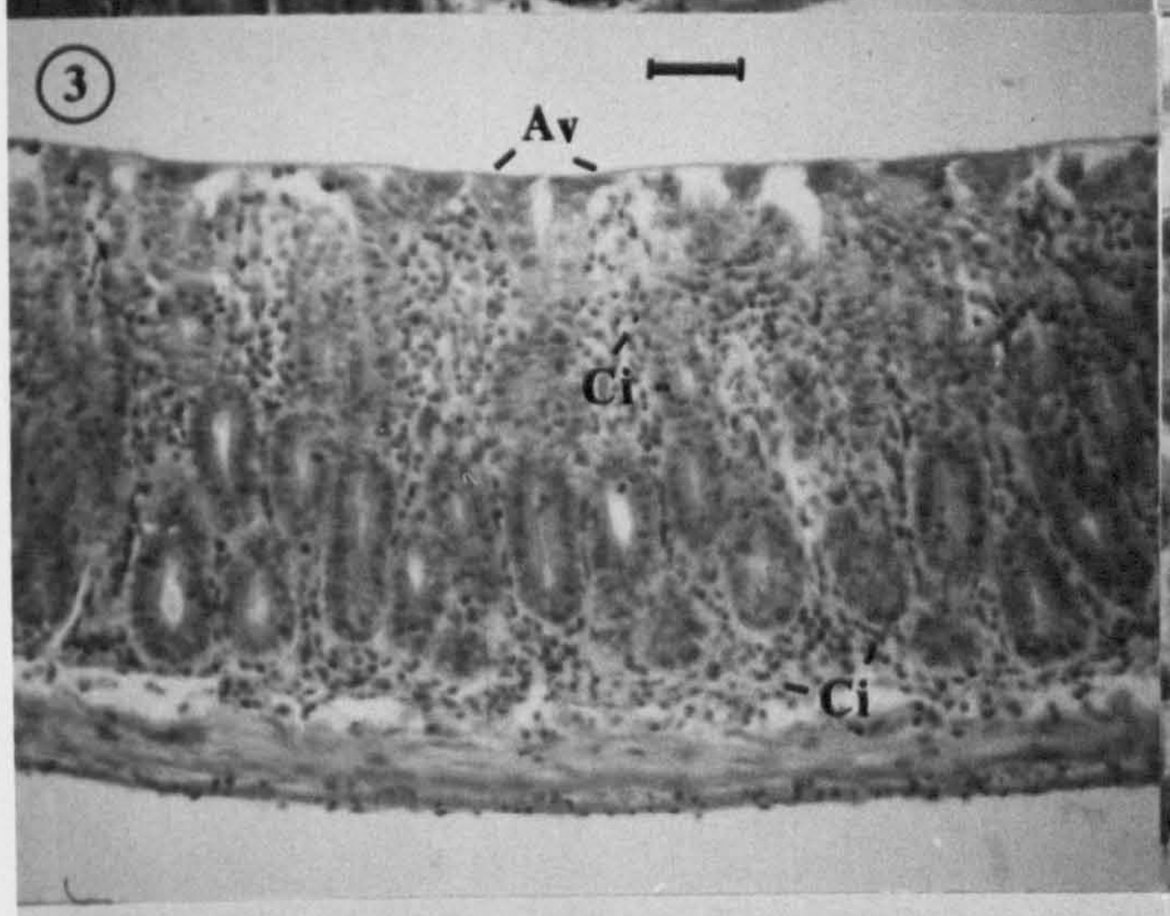
Tm-tunica muscularis P-lamina propria

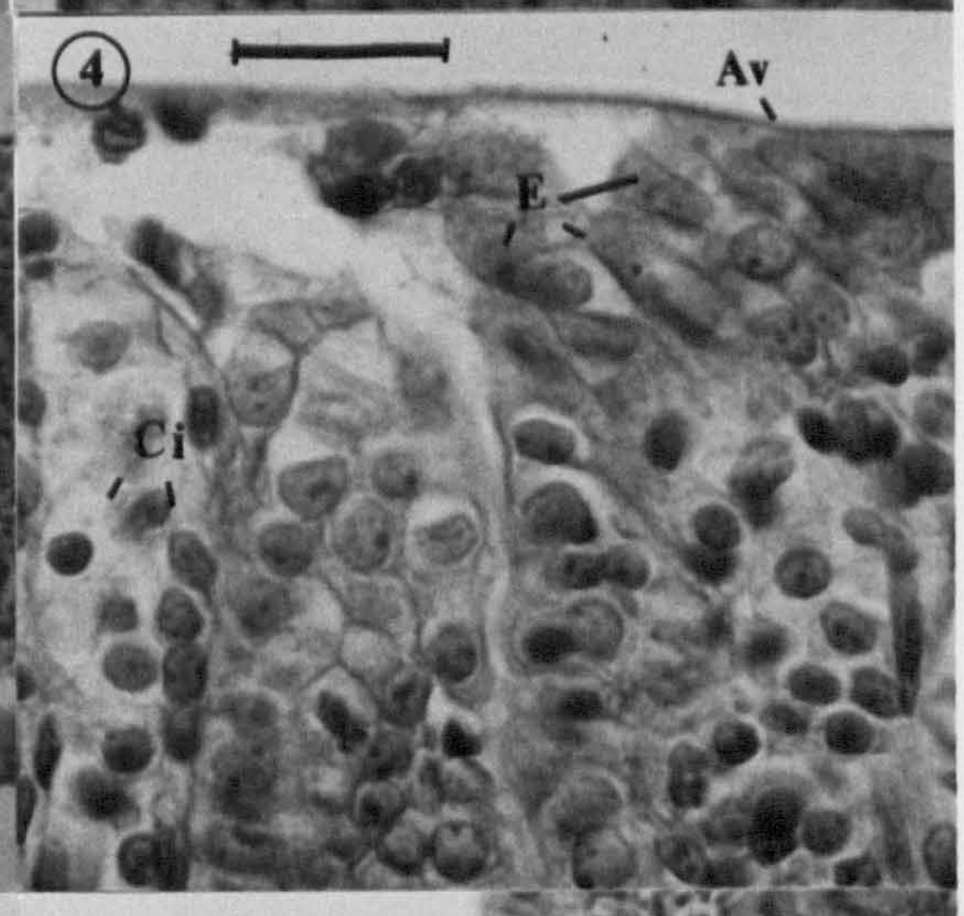
Ci-cellular infiltration Hc-hyperplastic crypts

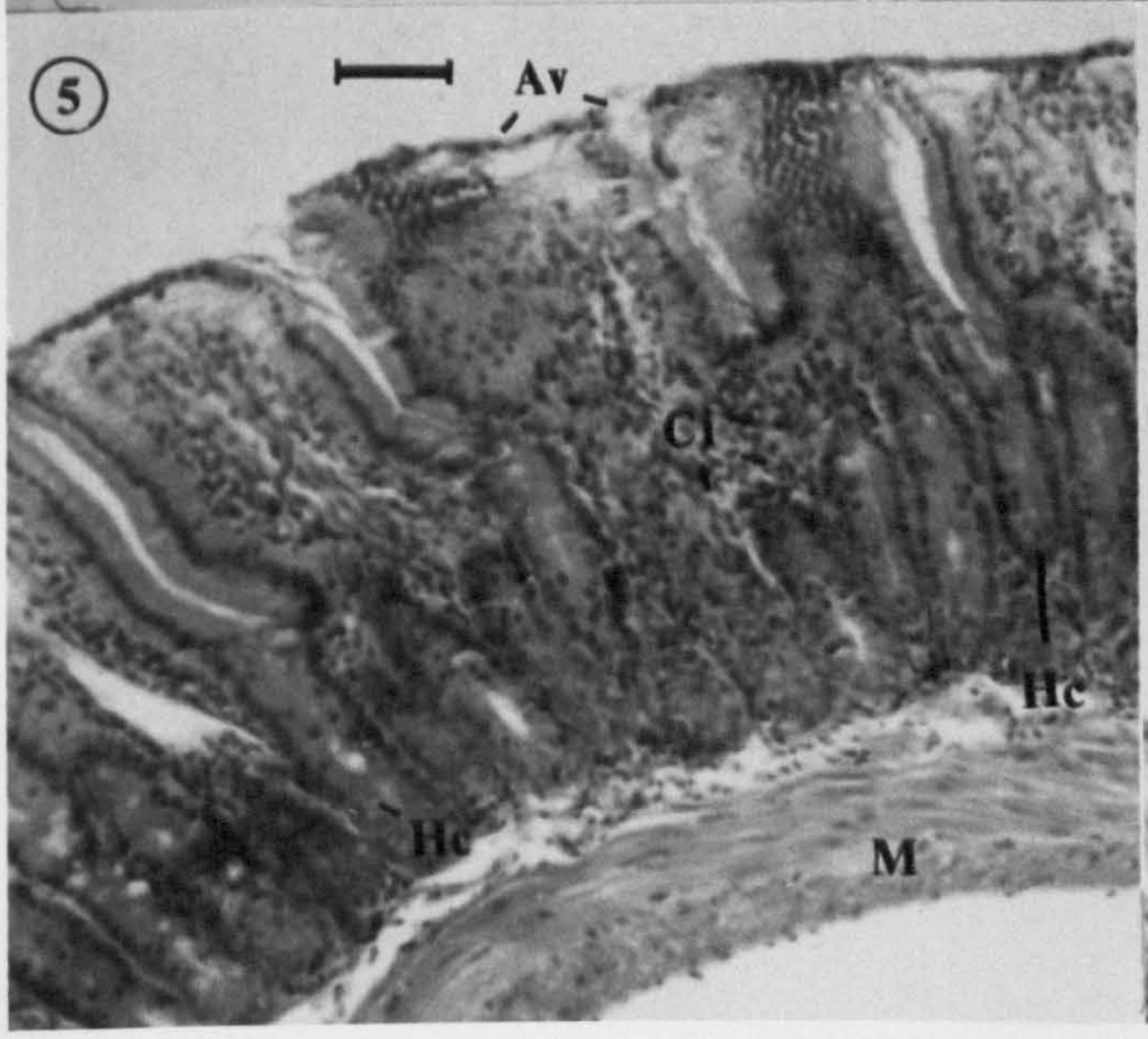
M-muscular hypertrophy Av-atrophic villi


1
V
E
P
C
Tm


2
B
P
E


3
Av
Ci


4
Av
E
Ci


5
Av
Ci
Hc
Hc
M

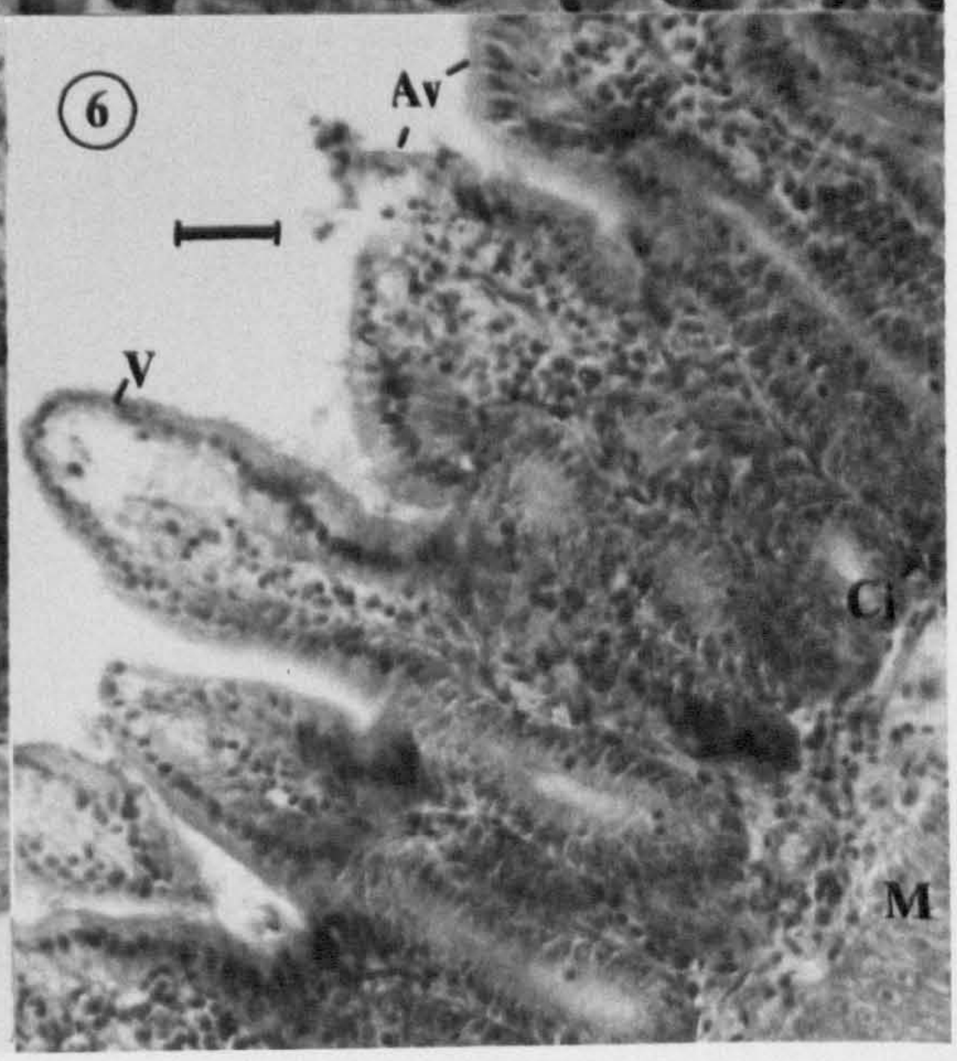

6
Av
V
Ci
M

Fig. 6.16 (see Section 6.3.10)

(1) Hyperplastic crypts and eroded villi

Scale bar= 50 μm

(2) Region of small intestine containing Peyers patch. Note lymphocytic infiltration atrophic villi and hyperplastic crpyts

Scale bar= 100 μm

(3) Severe villus erosion

Scale bar= 100 μm

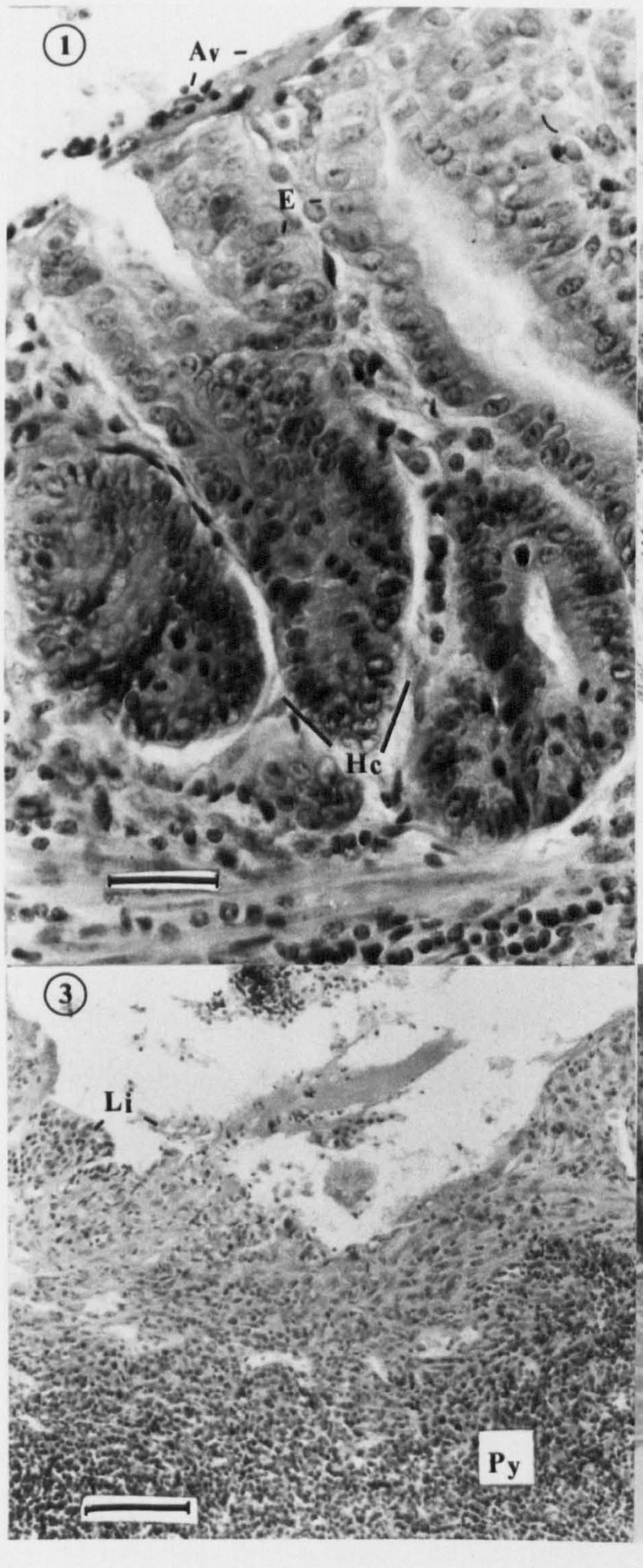
(4) Hyperplastic crypts. Note the much enlarged tunica muscularis

Scale bar= 50 μm

Key

Av-atrophic villi Hc-hyperplastic crypts

Py-Peyers patch Li-lymphocytic infiltration


1
Av
E
Hc
2
Av
Li
Hc
Li
Py
3
Li
Py
4
Hc
M

muscularis is evident, though this is not easily observable as a gross external change in the appearance of the gut serosal surface after the gut has been exposed during dissection. Fig 6.15 (6) is the main region of attachment of worms in a small intestine of a mouse exposed to 25 metacercarial cysts and harbouring 14 worms, at 24 days postinfection. Partial erosion of the villi is evident along with muscular hypertrophy, the formation of hyperplastic crypts and cellular infiltration. Interestingly, the remains of a single shortened villus are seen protruding into the gut lumen.

Fig 6.16 (1) is a region of jejunum of a mouse exposed to 100 metacercarial cysts and harbouring 32 worms at 20 days postinfection close to the main site of infection. Fig 6.16 (2) is a low power view of this area containing a Peyer's patch. Infection has lead to the formation of atrophic villi and hyperplastic crypts resulting in a high crypt to villus ratio (approx. 5.1). This view of the Peyer's patch shows considerable lymphocytic infiltration. Fig 6.16 (3) shows a region of the small intestine containing a Peyer's patch from a mouse exposed to 100 metacercarial cysts and harbouring 33 worms at 12 days postinfection close to the main site of worm attachment. There has been a severe disruption of villus structure and marked lymphocytic infiltration. Fig 6.16 (4) is an area of the jejunum from a mouse exposed to 100 metacercarial cysts and harbouring 50 worms at 24 days postinfection at a main site of worm attachment. The already-described pathological features are noticeable along with the much enlarged tunica muscularis

giving rise to a marked muscular hypertrophy that was visible in this instance at the gross pathological level.

6.4 Discussion

6.4.1 Worm establishment

The proportional establishment success of *E. liei* in the final host appears to be dependent to a degree on the number of metacercarial cysts administered to Swiss T.O. male mice. The negative linearity exhibited in the relationship between percent establishment success (over the period 8 to 24 days postinfection) and initial cyst exposure density suggests a density-dependent constraint on *E. liei* establishment in the mouse host. These results, although based on only a small number of mice, indicate that the overall percentage worm recovery is greater in mice administered low numbers of metacercarial cysts, (6), when compared with the higher cyst densities (25 or 100). In complete contrast to these findings, Odaibo, Christensen and Ukoli (1988) found that initial worm percentage establishment of *E. caproni* in NMRI mice infected with 6 and 25 metacercarial cysts was infection-dose independent, while Franco (1988) *et al.* found no relationship between establishment success of *E. revolutum* in hamsters and cyst exposure densities of 125, 300 and 500. Nollen (1983) demonstrated that percentage recovery rates of the digenean *Philophthalmus gralli* infections in the eyes of chickens were significantly higher in infections with 6 and 10 metacercariae per eye than in infections with 100 per eye. A positive linearity in relation to the percentage number of worms recovered in increasing infection intensities has been demonstrated by

Flavell, Flavell and Field (1983). These workers infected golden hamsters with varying cyst densities of the liver fluke *Opisthorchis viverreni* and concluded that worm recovery rates were dependent on the number of metercercariae given in that the percentage worm establishment was greater in hamsters infected with 50 or more metacercarial cysts and less in those administered 10 or 20 metacercarial cysts. They were unable to state any reasons for the direction of this apparent density-dependent relationship.

On the other hand, non-digenetic host-parasite systems have revealed a similar relationship to that observed in the present study. Intestinal nematode systems have provided clear evidence for density-dependent relationships as demonstrated by Michael and Bundy (1989). With regards to establishment success they found that the relationship in CBA/Ca mice infected with varying densities of the nematode *Trichuris muris* implied a dose-dependent constraint on *T. muris* establishment in its mouse host. Although little is known about the phenomena surrounding establishment success in relation to density and its evolutionary significance, parasite self-regulation and within-host factors such as dimensions of the mouse intestine probably play a major role. Beaver (1980) has suggested a physical limitation on gut carrying capacity for the human nematode *Ascaris lumbricoides* which implicates a role for parasite size in the process of population regulation.

6.4.2 Gut microhabitat use by *E. liei* in relation to density

The mean positions of populations of *E. liei* in the mouse small intestine following varying metacercarial cyst exposure densities imply that exposure density has no marked effect on worm positions. As the infection densities increase the greater number of worms that become established means that the spatial utilization of the worms will spread because of the carrying limitations of sections of the small intestine of the mouse. Even in this situation however, *E. liei* is confined exclusively to the small intestine. The majority of the worms at each infection level inhabit the 50% to 80% region of the gut up to day 24. The ileum and jejunum then are the regions utilized by the worm populations over this time period. The jejunum being the main parasitized region of all the infection densities (also revealed by the histopathological studies). From observations on the spatial distribution of the worms following various exposure densities it is apparent that all worm populations utilize the same intestinal regions. Odaibo *et al.* (1988) found that in NMRI mice infected with 6 metacercarial cysts of *E. caproni,* worms migrated anteriorly with worm localization in the 20% to 80% region of the small intestine on days 14 and 21. In contrast, worms in infections of 25 metacercariae remained in the 60% to 100% region. Franco *et al.* (1988) infected golden hamsters with 125, 300 and 500 metacercarial cysts of *E. revolutum* and noted that the majority of the worms in the 300 and 500 exposure cyst infection were recovered from the duodenum, jejunum and ileum whereas worms from the 125 cyst infection were mainly found in the

jejunum. Franco *et al.* (1988) commented that increased dosage levels of metacercarial cysts enlarged the spatial distribution of the parasites in the gut.

6.4.3 The effect of cyst exposure density on body dimensions

Increased metacercarial cyst exposure density seemed to have no consistent effect on width and the length of *E. liei* in the mouse small intestine. Franco *et al.* (1988) multiplied the length by the width of worms of *E. revolutum* infections at metacercarial cyst densities of 125, 300 and 500 to obtain a projected first order estimate of the body area. They, in contrast to the present findings, found that there was a significant difference between the estimated body areas of infection dose groups exposed to 125 and 500 metacercarial cysts at days 7, 14, 28 and 35 but no significant difference at day 21 between any of the infection dose groups. Odaibo *et al.* (1988) carried out the same calculation and found no significant differences in the area of *E. caproni* in density 6 and 25 infections of *E. caproni* in NMRI mice. Mohandas and Nadakal (1978) found that increasing the numbers of *E. maylayanum* cysts used to infect rats reduced the length of the parasite in the intestine. Worms of *E. maylayanum* recovered from rats infected with 500 and 800 metacercarial cysts were significantly smaller than worms recovered from rats infected with 10 metacercarial cysts. From the very high initial metacercarial cyst dose levels used by Mohandas and Nadakal (1978) and Franco *et al.* (1988) it may be deduced that the effects of density on growth in members of the genus *Echinostoma* occur only at very high infection levels.

Other digenetic studies such as that of Flavell *et al.* (1983) noted the decrease in length of *O. viverrini* as the worm burden increased in hamsters. These effects have also been seen in *Fasciola hepatica* in cattle (Ross, 1965) and the development of the liver fluke *Fascioloides magna* in sheep (Foreyt and Todd, 1976). The classical study carried out on intraspecific crowding of intestinal gut dwelling parasites was conducted by Roberts (1961) using the cestode *Hymenolepis diminuta* grown in rats. He found a clear negative relationship between worm population density and worm length.

The main underlying mechanisms of these size-related density phenomenon are probably simple mechanical constraints and competition for limited nutrients. Theories have been suggested by Fischthal, Carson and Vaught (1982) and Roberts and Insler (1982) which assume that chemical "factors" released by worms may be important negative influences on worm growth. High worm densities would lead to higher and more potent concentrations of these chemical factors. A direct comparison of all the data related to the effects of density on helminth infections is confounded by the various experimental density ranges utilized and the biological differences that exist between the three distinct taxa, that is, the Digenea, Cestoda and the Nematoda. On the whole, growth in helminths is genetically determined (Smyth and Halton, 1983) and in relation to nematodes, mature worms are constrained by a cuticle which physically prevents much further growth once maturity is reached (Chappell, 1980). Digeneans are different from both

nematodes and cestodes since they do not possess a limiting cuticle like the nematodes and neither do they possess the considerable growth potential that cestodes possess in the form of proglottids which ensures that cestodes have the ability to carry out rapid increases in growth over a short space of time. Cestodes have the ability to carry out destrobilation whereby the majority of the proglottids are shed leaving only the scolex and the head attached to the mucosal surface (Chappell, 1980). In this respect it would seem apparent that the effects of worm density on size are more easily detectable in the cestodes which possess the mechanism for both rapid growth and destrobilation and thus subsequently, possess a marked ability for size regulation. In such circumstances it is therefore not surprising that the effects of density on size are easily identifiable in cestode infections as demonstrated by Roberts (1961) and Hesselberg and Andreassen (1975) with *H. diminuta* and Halvorsen and Andersen (1974) in *Diphyllibothrium dentriticum*.

6.4.4 The effect of cyst density on *in-utero* egg counts

It is evident from Fig 6.5 and 6.6 that initial cyst exposure density does have an effect on the number of eggs recovered from the uteri of worms recovered from the resulting infections. The effects of this process are most noticeable at days 20 and 24 postinfection in the infections produced by 100 cyst exposures when compared with the lower infection levels. This density-dependent constraint appears to reduce the carrying capacity of the uteri of worms in higher density infections. It is not clear at present whether this reduction is a consequence of an overall

lower rate of egg production, a smaller uterine volume, a feature linked wilh uterine translocation of eggs or some combination of these factors. The decrease in the number of *in-utero* eggs in relation to increasing density may be a direct consequence of the so called "crowding effect". A decrease in the number of eggs in this situation may imply that development of the uterus is delayed or hindered completely or its egg-carrying capacity retarded because of the marked operational density dependent constraints.

In-utero egg counts (and egg producing capability as a whole) as a means of monitoring the effects of density on echinostome infections has received very little attention, although Franco *et al.* (1988) did note that eggs appeared in *E. revolutum* at day 9 in the smaller infections (125 metacercarial cysts) but that the time when *in-utero* eggs were first visible was delayed in the heavier infections (500 cyst), appearing at day 10. This result was only noted from observation and no analysis carried out. Fried and Freeborne (1984) fed chicks 100 metacercarial cysts of *E. revolutum* and noted that at 3 weeks that worms from crowded sites (greater than 25 worms/site) contained about 250 eggs per worm, whereas those from non-crowded sites (1-10 worms/site) contained about 500 eggs per worm. These results of Fried seem to signify the variation in uterine egg counts that can occur within a population as a direct result of the aggregational behaviour of these worms.

6.4.5 Density-dependency and egg output per worm

Density-dependent constraints are also evident on the number of eggs produced per worm at each level of initial density infection. The results of this study reveal that egg production per worm is directly related to worm burden, egg output per worm generally decreasing in those populations carrying a greater burden of worms. This relationship is confirmed by the cumulative egg output per worm over successive days postinfection and the global total egg production per worm at each of the initial cyst exposure densities. These values being 30,567.09, 14,368.09 and 8,677.9 at initial cyst exposure densities of 6, 25 and 100 respectively, for the first 24 days of the infection. Interestingly, it can be seen (see Fig. 6.9) that egg output is reaching a plateau in the 100 cyst exposure density but still rising in the lower densities (particularly in the density 6 exposure group). Previous studies on *Echinostoma* models have failed to produce comparable data on cumulative egg output and worm density which could be compared with that produced in this study. Similar relationships involving egg ouput per worm however have been described by Flavell *et al.* (1983) in the digenean *O. viverrini* and for a number of helminths in man, notably *Schistosoma mansoni* and *Schistosoma haematobium*, (Cheever, Kamel, Elwi, Mosimann and Danner, 1977) and *Ascaris lumbricoides* (Croll, Anderson, Gyorkos and Ghadirian, 1982). Croll *et al.*, (1982) stated that such relationships may be generated both by finite resources within the host such as nutrients or space and by the immunological response of the host to parasite infection. None of these studies

however adressed the question of cumulative egg output by worms at different densities

What is clear however, is that there is a very strong correlation between uterine egg counts (EPU) and mean egg ouput per worm per 24 hours ($\bar{x}$EPW) in all stages of these experiments (see Fig. 6.10a). This in many ways is an encouraging result in the context of the many previous studies in a range of helminths which have assumed that the number of temporarily stored eggs in a uterus or pseudocoelom can be used as an indirect measure of egg production rate. The present findings provide one of the few examples of a positive confirmation that this assumption can be justified in some circumstances.

6.4.6 Possible mechanisms and consequences of density-dependent constraints on *E. liei*

There appears then to be a variety of ways in which worm density seems to impact on populations of *E. liei* in its mouse host. In particular, increasing worm intensity appears to reduce population establishment success, produce a decline in uterine egg numbers and a decline of similar magnitude in egg output per worm per 24 hours and cumulative egg production. It is interesting to note that one effect of the density-dependent phenomena is that by day 24 of the infection, worm populations at all densities are producing a total output per population per 24 hours of about 10,000 eggs.

Another way of assessing the magnitude of this density dependence is to consider the ratios of initial infection

exposures, that is 6:25:100 (that is, 1:4.2:16.7) and compare them with the estimated cumulative egg outputs per average mouse over 24 days of the infection. These latter figures are 128381.8, 178164.3 and 289841.9 respectively, a ratio of 1:1.4:2.3. These cumulative relative mouse egg outputs are massively depressed by increasing worm density. This density dependence probably has significance for *E. liei* population regulation indicating fecundity is a major regulatory force. Population stability is known to be a characteristic feature of helminth infections and is presumed to indicate that density-dependent mechanisms are operating (Keymer and Slater, 1987). Anderson and May, (1978) have explained that the regulation of helminth infections occurs primarily as a result of limitations imposed on the build up of parasite sub-populations within individual hosts. Such effects as seen in this study that are generated by increasing worm burdens can probably be ascribed to mechanical constraints and competition for optimum preferential sites amplified by aggregational behaviour (see Chapter 5). It is a possibility that host immunological responses may play a role that increases disproportionately in efficiency as parasite burden increases (Anderson and May, 1978, Keymer, 1982 and Anderson and May, 1985). Other workers (Wakelin and Wilson, 1980 and Lloyd and Soulsby, 1987) have implied that host immune responses both, humoral and cellular, depress both helminth growth and fecundity.

6.4.7 The pathological effects of density

Throughout the experiments described here, infected mice weights were comparable with non-infected controls. In

complete contrast Fried and Wilson (1981) noted that chicks infected with 50 to 75 cysts of *E. revolutum* necropsied at 14 days postinfection lost weight while Huffman *et al.* (1986) found that hamsters infected with over 45 worms of *E. revolutum* also lost weight over 28 days, in one instance a hamster which weighed approximately 145 g and had a worm burden of 67, lost 35 g in 21 days. Huffman *et al.*, (1988) also observed similar results in a later study. They noted that hamsters experienced weight loss infected concurrently with 100 metacercarial cysts of *E. revolutum* and 100 metacercarial cysts *E. liei* and also in single infections infected with 100 cysts of *E. revolutum* and 100 cysts of *E. liei* over 13 days postinfection. Fried and Wilson (1981) speculated that worm competition for host ingesta, interference with digestion and diminished absorbtion may be involved in the observed decrease in body weight. Huffman *et al.*, (1986, 1988) stated that decreases in body weight in hamsters were due to severe diarrhoea and consequent fluid loss. It seems evident that in the *E. liei*/Swiss T.O. mouse pairing, gross weight loss pathology of the type and magnitude described by all these workers in other systems does not occur.

From the present results it appeared that the PCV of hosts infected with metacercarial cyst exposure densities of 25 or 100 ultimately show a reduction when compared with PCV of control mice or mice infected with an initial 6 metacercarial cyst exposure. The initial increase in PCV in the metacercarial cyst exposure density 25 and 100 infections may imply the increased production of more red blood cells by the mouse at the onset of infection or the enlargement of the red blood cells, a decrease

may be as a result of haemorrhages along the length of the intestine evidence of such pathology being seen in the histopathological investigations here. Huffman *et al.*, (1986) found that there was an infection intensity-variation in relation to PCV as hamsters infected with 45 metacercarial cysts of *E. revolutum* showed a greater overall increase in PCV than those hamsters infected with less than 45 cysts but did not offer any hypotheses as to the causation of these changes. In the later study carried out by Huffman *et al.*, (1988) theses workers found that PCV values increased in infections with *E. revolutum* and concurrent infections with both *E. revolutum* and *E. liei* but that PCV decreased in *E. liei* infections. In both of these studies peripheral blood smears from infected hamsters were normal and in the present study no differences were revealed between the monocytes, lymphocytes and the granulocytic series of infected mice with control mice at the various cyst exposure densities examined.

6.4.8 Pathology resulting from *E. liei* infection

The present study has shown that in *E. liei* infections the histological pathogenic changes are not confined to the attachment sites which are traumatic in nature as are those described in cattle with acute amphistomiasis (Boray, 1959). Very pronounced changes were observed at all the infection levels which included aspects of the following :- villous atrophy, crypt hyperplasia, hypertrophy of the muscle layers, dilation of the gut and cellular infiltration. These features were common at the attachment sites and the adjacent surrounding areas. The overall pathological changes were on the whole indistinguishable

between exposure density levels, with the only significant difference apparently being that the effects were more marked in the cyst exposure density 100 infections, that the hypertrophy of the tunica muscularis increased with increasing time postinfection (and only in the highest density infections) and enlarged Peyer's patches were only seen near the main worm clusters in these high density infections.

These pathological changes that were found not to be confined to the site of attachment in *E. liei*, were similar to those reported for *E. revolutum* in mice (Bindseil and Christensen, 1984) *E. revolutum* in hamsters (Huffman, Michos and Fried 1986 and Mabus, Huffman and Fried, 1988) and *E. caproni* in chicks (Kim and Fried, 1989). Bindseil and Christensen (1984) observed in *E. revolutum* infected mice, crypt hyperplasia, villous atrophy and subepithelial fibrosis as the most conspicuous features along with a hypertrophy of the muscle layers. They found that these changes were thymus-independent as they were found to be as severe in athymic mice as in conventional mice. Huffman, Michos and Fried (1986) as well as noting these same pathological features in the small intestine also observed *E. revolutum* in the liver. They observed suppuratve lesions in the liver produced by bacterial infections which they believed to have been due to the transporting of intestinal flora by the parasite as it moved up the bile duct on its way to the liver. A further study involving *E. revolutum* in the golden hamster noted the occurence of this parasite in the liver, gall bladder, pancreas and the stomach (Huffman, Iglesias and Fried, 1988). This spread of site utilization by the echinostomes was

attributed by the authors to worm crowding in heavy infections after hamsters were exposed to 350 metacercarial cysts of the parasite. Extraintestinal infection resulted in marked necrosis and haemorrhage caused by the parasite in the liver parenchyma and the biliary system.

Huffman, Alcaide and Fried (1984) explained that the histopathological response of *E. liei* and *E. revolutum* in single and concurrent infections in hamsters showed the erosion of intestinal villi with lymphocytic infiltration as the primary response. Mohandas and Nadakal (1978) reported that the pathological changes in the rat intestine caused by *E. malayanum* included lysis and destruction of mucosa, increased activity of goblet cells, oedema and reticulated appearance of lamina propria and hyperplasia of epithelial cells. Haque and Siddiqui (1978) observed the histopathology of *E. maylayanum* infections in pigs and noted damage to the cells of the intestinal epithilium and to the villi but saw no intestinal haemorrhage in the vicinity of the parasites. Echinostomiasis has also been reported in ducks parasitised with *E. revolutum* (Kishore and Sinha, 1982). They observed the erosion of the mucosal epithelium and that villi, that had been drawn into the ventral suckers showed pressure atrophy and desquamation of epithelial cells. It is well documented that intestinal digeneans attach to the mucosa of their hosts with their ventral sucker (Boray, 1959; Kishore and Sinha 1982; Smyth and Halton,1983). Simonsen, Christensen and Koie, (1989) have demonstrated that *E. caproni* attaches to the mouse small intestine by grasping a plug of mucosa with its ventral sucker. The densely compressed

villi occupy the inner cavity of the ventral sucker. This investigation showed that these attachment sites are elevated areas of mucosa that are only temporary features formed by the mechanical grasp of the ventral suckers. These workers stated that these elevated attachment sites disappeared after mechanical removal of the worms. This mechanism is possibly operative in the present study as pathology at the sites of attachment left some villi extending into the gut lumen above villi that had been eroded, a pattern that was not observed in adjacent areas.

The hypertrophy of the muscular layers as well as being observed in echinostome infections has also been observed in other parasitic conditions such as nippostrongyliasis (Symons, 1957). This hypertrophy appears to occur at the same time as a dilation of the diameter of the intestine associated with a collection of inflammatory fluids in the lumen. A similar intestinal inflammatory response in hamsters infected with *E. revolutum* (Mabus *et al.* 1988) was coupled with prominent lymphocytic infiltration of the villi. Bindseil and Christensen (1984) reported that, in conventional mice, the infiltrates consisted of lymphocytes, eosinophils and plasma cells which were seen in the lamina propria as well as in the submucosa. A similar infiltration occurs in the *E. liei* infections of mice except that lymphocytes appear to be the main component of the infiltrate. Lymphocytic and monocytic infiltration has been recorded in certain echinostome infections as being the primary response to infection (Kishore and Sinha, 1982; Bindseil and Christensen, 1984; Huffman *et al.* 1986; Huffman *et al.*, 1988).

Bindseil and Cristensen (1984) noted that only in athymic mice were the cellular infiltrations dominated by eosinophils. It is believed there is a marked association between helminth infections and increased levels of circulating eosinophils (Spry, 1988).

Crypt hyperplasia and villous atrophy are common to many different clinical conditions such as coeliac disease in man (Shiner and Doniach, 1960) *Nippostrongylus brasiliensis* infections in rats (Symons and Fairbairn,1963) and giardiasis in mice (Roberts-Johnson and Mitchell,1978). Ferguson and Jarrett (1975) suggested that a single effector mechanism may cause these morphological changes after providing evidence that these features in nippostrongyliasis in rats were thymus-dependent. As Bindseil and Cristensen (1984) have shown the pathological changes in *Echinostoma* to be thymus-independent this hypothesis is obviously not applicable in all cases. It is possible to speculate that the formation of atrophic villi may be partly due to direct mechanical damage caused by the cephalic spines and the ventral and oral sucker of *E. liei* as a result of feeding activity. The villus epithelium appears to be a principal site of *E. liei*-induced damage and the crypt hyperplasia could well be a compensatory mechanism to counteract the accelerated loss, as a result of parasitisation, of damaged villus epithelium. The increase in the crypt to villus ratio was also noted by Bindseil and Christensen (1984) in both conventional and athymic mice and is obviously a product of hyperplasia and villus shortening.

Feeding in digeneans is a complex and poorly understood phenomenon, with much of our knowledge based on a small subset of species including *Schistosoma mansoni* and *Fasciola hepatica* and members of the Strigeoidea (Halton and Smyth, 1983). It has been long known that schistosomes ingest host red blood cells which are haemolysed rapidly within the lumen of the intestinal caecae (Jennings, 1968). It has been suggested that *Fasciola hepatica* browses upon the hyperplastic epithelium of the infected bile duct (Dawes, 1963) and is also a blood feeder (Halton,1967). It is now believed that *Fasciola* is primarily though not exclusively a blood feeder (Halton and Smyth, 1983). Sukhdeo, Sangster and Mettrick, (1988) have since hypothesised that *Fasciola hepatica* grown in rabbits feeds only on blood from haemorrhagic ulcers in the hyperplastic mucosa of the infected bile duct. Fried and Caruso (1970) have reported that *E. revolutum* adults from the chick feed on mucosal tissue and incidentally on blood. Wisenewski, Fried and Halton (1986) stated that *E. revolutum* grown in chicks fed on the host intestinal mucosa and that worms of *E. revolutum* grown on the chick chorioallantois fed on blood. Ultrastructural examinations of the luminal contents of worms grown in the chick, revealed the presence of portions of membrane and myelin figures, scattered vesicular elements and clumps of heterochromatin but no evidence of blood cells. Examination of the luminal contents of worms grown on the chick chorioallantois revealed the presence of a mixture of nucleated erythrocytes in various stages of disintegration, together with granular portions of freed haemoglobin, fragments of membrane and lipid-like droplets. With this in mind it may be presumed that *E. liei* has the

capability to be both a blood and tissue feeder. The presence of areas of mucosal haemorrhage and endothelial damage without closly associated worms in experimental mouse infections and the worms apparent ontogenetiic migrations (see Chapter 5) suggest that groups of *E. liei* feed on mucosal material in a group and then move to a less damaged area to continue feeding. It is possible that the damage enables nutritionally valuable material such as blood and low molecular weight organic substances to leak into the gut lumen where they can be utilized by the worms.

These pathological changes could also have been partially induced by host immune reactions to the parasite. It has been demonstrated in mice that infections with *E. caproni* induce a serum antibody response to the surface of the parasites (Simonsen and Andersen, 1986). These surface-bound antibodies were found to be rapidly lost when these worms were grown *in vitro*. This lead these workers to suggest that this could be due to the shedding of surface antigen which could possibly be an adaption to withstand the host's immune attack. Andersen, Simonsen, Andersen and Birch-Andersen, (1989) also demonstrated the release of antigen from the surface of *E. caproni* cultured *in vitro*. The enlargement of Peyer's patches seen in the metacercarial cyst exposure density 100 infections may be the result of a cellular response initiated by the arrival of worm antigens at these lymph nodes in close proximity to worm aggregates.

It has been suggested that some or all of these pathophysiological changes are directly or indirectly generated by parasite produced vasoactive intestinal polypeptide (VIP)-like material (Thorndyke and Whitfield, 1987). These workers identified a sub-population of VIP-like immunoreactive tegumentary cells in 12 day old worms of *E. liei* from Swiss T.O. mice which putatively could release the peptide into the worm's immediate surroundings and induce changes in the adjacent gut tissues. This aspect will be considered in greater detail in the following Chapter.

CHAPTER 7

VERTEBRATE-LIKE NEUROPEPTIDES AND THE DEVELOPMENT OF *ECHINOSTOMA LIEI*

7.1 Introduction

Peptides have long been recognised as having important regulatory roles in the brain and gastrointestinal tract of many vertebrates, as well as being known to have crucial physiological functions (Bloom, 1977). They have been thought to act in three major ways in vertebrates as peptidergic neurotransmitters, as locally acting paracrine system substances and as circulating hormones (Bloom and Polak, 1978). It is now assumed that peptides have a taxonomic distribution in animals that includes the invertebrates as well as the vertebrates. Their overall role in the invertebrates has been defined as one of involvement in central and peripheral nervous transmission (Falkmer, Hakanson and Sundler, 1984), while Greenberg and Price (1983) have stated that there are two types of invertebrate neuropeptides. The first type, they believe, are native to the invertebrates and have been identified by their ability to modify or regulate known physiological or biochemical processes in invertebrates. Examples of these peptides include molluscan cardioexcitatory tetrapeptide (FMRFamide), which is involved in muscle contraction and relaxation in molluscs (Price and Greenberg, 1980) and adipokinetic hormone (AKH) which in locusts is involved in lipid mobilisation (Stone, Mordue, Broomfield and Hardy, 1978). The second type of invertebrate peptides, which they termed naturalized, have been discovered more recently and are identified by their binding capacity to antisera raised to known vertebrate peptides. Examples of this second type include substance P-like immunoreactivity indentified in the nervous system of *Hydra*, (Grimmelikhuijzen, Balfe, Emson, Powell and

Sundler, 1981) bombesin-like immunoreactivity also demonstrated in the nervous system of *Hydra* (Grimmelikhuijzen, Dockray and Yanaihara, 1981) and somatostatin and substance P-like immunoreactivity, identified in the neural ganglion of the tunicate, *Ciona intestinalis* (Fritsch, Van Noorden and Pearse, 1979). These types of findings led Greenberg and Price (1983) to conclude that vertebrate peptide families have homologues distributed throughout the invertebrate phyla. Thorndyke (1986) has suggested that peptide regulators were utilized in multicellular animals as messengers in the nervous system. He explained that they have probably persisted throughout the animal kingdom because some peptide sequences make particularly useful messengers and have therefore been retained within the genome of probably all higher and lower animals to be drawn upon when selective pressures demand. Grimmelikhuijzen (1984) has established the presence of a number of neuropeptides in the nervous system of the coelenterate, *Hydra*. Interest in other peptide regulators in other lower vertebrates such as the annelids and platyhelminths began when Sundler, Hakanson, Alumets and Walles (1977) demonstrated the immunochemical reactivity of pancreatic polypeptide PP-like and vasoactive intestinal peptide VIP-like material in the nervous system of *Lumbricus terrestris*. Bombesin-like, gastrin/CCK-like and substance P-like immunoreactivities have been additionally demonstrated in the leech, *Hirudo medicinalis* (Osborne, Patel and Dockray, 1982).

The immunocytochemical reactivity of neuropeptides has also more recently been demonstrated in a number of parasitic helminths. Gustafsson (1987) demonstrated neuropeptide immunoreactivity in the nervous system of adult *Schistosoma mansoni*. Davenport, Lee and Issac (1988) provided an immunocytochemical demonstration of FMRFamide-like immunoreactivity in the nematode *Ascaris suum*, Fairweather, Macartney, Johnston, Halton and Buchanan (1988) demonstrated various vertebrate-like neuropeptides (PP, peptide tyrosine tyrosine PYY, peptide histidine isoleucine and gastrin releasing peptide) in the nervous system of excysted cysticercoid larvae of the rat tapeworm *Hymenolepis diminuta*. while Maule, Halton, Johnston, Fairweather and Shaw (1989) similarly demonstrated the presence of PP, PYY, FMRFamide and VIP-like immunoreactivities in the fish-gill parasite *Diclidophora merlangi*. These studies have highlighted the involvement of neuropeptides within the central and peripheral nervous system of parasitic helminths and it is probable that these location sites can provide a guide to the function of these neuropeptides, in the context of their role as neurotransmitters.

Within *Echinostoma* species themselves, studies involving the immunocytochemical demonstration of vertebrate like peptides have been confined to *E. liei* (Thorndyke and Whitfield, 1987; Riddell, Whitfield, Balogun and Thorndyke, 1991) and *E. caproni* (Richard, Klein and Stoeckel, 1989). Richard *et al.* (1989) detected substance P-like immunoreactivity in the central and peripheral nervous structures and in the glandular cells of the prostate but did

not make suggestions on the peptide's possible functions. This pattern of localisation suggests that in the echinostomatid, the putative substance P is potentially operating both as a neurotransmitter (in the central and peripheral nervous system) and as a locally acting neurohormone (in the male reproductive system). Riddell, Whitfield, Balogun and Thorndyke (1991) demonstrated the presence of FMRFamide-like immunoreactive cells in the central nervous system and reproductive system of *E. liei*. The nervous distribution of this peptide suggested a role as a peptidergic neurotransmitter while its localization within the reproductive system pointed to a direct involvement with copulation acting either locally or as a neurotransmitter. Thorndyke and Whitfield (1987) identified VIP-like immunoreactivity but located the presence of this immunoreactivity in the tegument of *E. liei*. While other work has confirmed on numerous occasions the presence of peptide regulators in the nervous systems of both free-living (Schilt, Richoux and Dubois, 1981) and parasitic platyhelminths (Reuter, Karhi and Sehot, 1984; Gustafsson, 1987; Davenport, Lee and Issac, 1988; Maule, Halton, Johnston, Fairweather and Shaw, 1989), Thorndyke and Whitfield demonstrated, for the first time in an endoparasite, VIP-like immunoreactivity in a subpopulation of tegumentary cells.

This novel finding of immunoreactive cells in this particular site led these workers to speculate on a possible role in host pathophysiology linked with postulated secretion of the peptide into the gut lumen of the host. This work was carried out on adult

worms, 12 days postinfection. As the present study (Chapter 6) has revealed host pathology in infected mice with 8 day old worms and taking into account the Thorndyke and Whitfield hypothesis, this Chapter describes work undertaken to examine and evaluate the developmental association, if any, between neuropeptide immunoreactivity and increasing parasite age. The prime objective of this study was to see if it was possible to characterize the specific changes in neuropeptide secretions in *E. liei* during development. To accomplish this the peroxidase-anti-peroxidase (PAP) technique of indirect labelling (Sternberger, 1974) was employed, a modification of the peroxidase-labelling technique devised by Nakane and Pierce (1967). The presence of two neuropeptides was sought, VIP and secretin. The former was studied because of the previous (Thorndyke and Whitfield, 1987) demonstration of VIP-like immunoreactivity in 12 day old worms of *E. liei*. Secretin immunoreactivity was investigated because preliminary investigations with a wide range of antisera had suggested that immunoreactivities to this peptide might be present (M.C. Thorndyke personal communication).

VIP was originally isolated from porcine duodenum (Said and Mutt, 1970) and is a 28 amino acid peptide that has been shown to be structurally and biologically related to both secretin, glucagon and gastric inhibitory peptide (Mutt and Said, 1974) and is known to occur throughout the gastrointestinal tract and central nervous tissue of humans. It has been found from the lower oesophagus to the colon and rectum (Polak, Pearse, Garaud, and Bloom, 1974), in

the pancreas (Buffa, Capella, Solcia, Feigerio and Said, 1977) and in central and peripheral neurons (Larsson, Fahrenkrug, Schaffalitzky de Muckadell, Sundler, Hakanson and Rehfeld, 1976). Secretin is a basic polypeptide of 27 amino acids and an evolutionary origin from a common ancestral hormone for secretin, glucagon, VIP and gastric inhibitory peptide (GIP) has been proposed by Dockray (1977). They are known together as the glucagon family (see Fig. 7.1). Secretin-producing cells are predominantly found in the mucosa of the human duodenum and upper jejunum as well as of the stomach (Chey, Rhodes and Tai, 1978).

7.2 Materials and Methods

7.2.1 Culture of *E. liei*

Metacercarial cysts of *E. liei* were dissected from the pericardial cavities of *Biomphalaria glabrata* snails. These cysts were then used to infect two groups of 8, 6-week old male Swiss T.O. mice. Each mouse was orally administered, 50 metacercarial cysts of *E. liei* and provided food and water ad libitum (see Section 2.7).

7.2.2 Preparation of worm material for immunocytochemical treatment

From 4 to 11 days after exposure to metacercarial cysts 2 mice were necropsied on each day postinfection. The small intestine was rapidly removed and placed onto a cork-bottomed dish. Using a needled syringe, fresh aqueous Bouin's fixative (see Appendix 2) that had been made up immediately before necropsy was squeezed

Fig. 7.1 Amino acid sequences of secretin, VIP, glucagon and GIP (data from Bloom, 1977).

```
                                5                   10                  15                  20
SECRETIN:  HIS-SER-ASP- GLY-THR-PHE-THR-SER-GLU-LEU-SER-ARG-LEU-ARG-ASP-SER-ALA-ARG-LEU-GLN-ARG-

VIP :      HIS-SER-ASP- ALA-VAL-PHE-THR-ASP-ASN-TYR-THR-ARG-LEU-ARG-LYS-GLN-MET-ALA-VAL-LYS-LYS-

GLUCAGON:  HIS-SER-GLN- GLY-THR-PHE-THR-SER-ASP-TYR-SER-LYS-TYR-LEU-ASP-SER-ARG-ARG-ALA-GLN-ASP-

GIP :      TYR-ALA-GLU- GLY-THR-PHE-ILE-SER-ASP-TYR-SER-ILE-ALA-MET-ASP-LYS-ILE-ARG-GLN-GLN-ASP-

                   25                                                                  40
SECRETIN:  LEU-LEU-GLN- GLY-LEU-VAL-(NH2)

VIP :      TYR-LEU-ASN- SER-ILE-LEU-ASN-(NH2)

GLUCAGON:  PHE-VAL-GLN- TRP-LEU-MET-ASN-THR

GIP :      PHE-VAL-ASN- TRP-LEU-LEU-ALA-GLN-GLN-LYS-GLY-LYS-LYS-SER-ASP-TRP-LYS-HIS-ASN-ILE-THR-GLN
```

into the proximal and distal open ends of the small intestine. This was carried out until the whole length of the intestine became filled with the Bouin's fixative. The small intestine was then opened with a longitudinal incision and pinned out along its whole length. The small intestine was then searched for worms. Adult worms, that is sexually mature worms from 8 days-old onwards, were visible with the naked eye. Worms younger than this were identified with the aid of a binocular microscope. Individual worms were then removed from the small intestine and transferred to fresh cold Bouin's fixative and fixation was continued for 24 hours at 4° C. The total number of worms recovered on each day from each mouse were mixed and treated as one sample.

7.2.3 Preliminary preparation of worms

After the initial 24 hour fixation, worms were transferred to fresh Bouin's fixative. The worms were then taken through a graded series of alcohols (70%, 90%, 96% and 100%). Worms were dehydrated for 20 minutes in each of the increasing alcohol concentrations but received an additional 20 minutes in fresh 100% alcohol. Worms were then finally dehydrated for 10 minutes each in two changes of xylene. The worms were then impregnated with molten paraffin wax (m.p. 57° C) for 20 minutes. After this treatment worms were embedded in paraffin wax and serial sections were cut using a rotary microtome, parallel to the worm's longitudinal axis. These serial sections were then transferred to glass slides that had a thin film of 0.4%(w/v) aqueous poly-L-lysine (Sigma) placed on their surfaces. These slides were then placed on a

hot plate set at 40° C to fix the sections onto the slides, for approximately 20 minutes.

7.2.4 Attachment of primary antibody

Sections so treated were immersed in xylene for 3 minutes and taken through a decreasing series of alcohols solutions as described above receiving 2 minutes in each solution. After the final wash in 70% alcohol the sections were completely immersed in freshly prepared phosphate-buffered saline (PBS) pH 7.2 (see Appendix 2) for 5 minutes, removed and placed in a second solution of fresh PBS (pH 7.2). A well-characterised primary antibody raised in rabbits against porcine VIP, #6 (Walsh), which is specific for the N-terminal region of VIP was used at a dilution of 1:200 with PBS (pH 7.2). Using a micropipette this primary antibody was then used to cover a number of individual sections on slides. The remaining slides received antiserum raised to natural porcine secretin (Thorndyke) at a dilution of 1:50 with PBS (pH 7.2) which was administered in the same way to worms at each time interval. The sections were then incubated in a humid chamber at room temperature for 24 hours.

7.2.5 Attachment of the secondary antibody (peroxidase conjugate)

After 24 hours worms were placed in 0.05M Tris (hydroxymethyl) amino methane -hydrochloric acid buffer (Tris-HCl) at pH 7.2 (see Appendix 2) for 1 minute. Using the same methodology as described for the primary antibody, the secondary goat anti-rabbit

serum conjugated to peroxidase (Dako Ltd.) was pipetted onto the sections in a 1:20 dilution with PBS. These slides were then incubated as before in a humid incubating chamber at room temperature for 1 hour.

7.2.6 Attachment of the tertiary antibody (peroxidase-antiperoxidase complex)

After the secondary incubation the sections were completely immersed in Tris-HCl buffer (pH 7.2) for a period of 1 minute. The sections were then removed and excessive amounts of buffer were carefully removed using filter paper. The subsequent and final antibody layer comprised rabbit peroxidase anti-peroxidase (PAP) complex (Dako Ltd.) which was added at a dilution of 1:80 with PBS, to the sections using the same procedure described for the primary and secondary antibodies. The sections were incubated as before for 30 minutes and then immersed in Tris-HCl buffer (pH 7.2) for 2 minutes and then transferred and washed in Tris-HCl buffer (pH 7.6) for 3 minutes.

7.2.7 Development of the peroxidase

The peroxidase was developed and visualised by immersing the sections in 0.06% 3,3 diaminobenzidine (DAB) and 0.01% hydrogen peroxide in 0.05M Tris-HCl (pH 7.6) for a maximum of 5 minutes. This substrate medium was made up immediately before use. After 5 minutes the sections were then removed from the DAB/Tris-HCl buffer (pH 7.6) and washed in tap water for 5 minutes. Subsequent treatment of the sections involved dehydration through an

increasing series of alcohols to xylene receiving 3 minutes in each solution. Finally the slides were mounted in the mounting medium DPX.

7.2.8 Controls

Specificity controls included omission of the primary antibody from a number of sections at each time interval and the preabsorption of the primary antibody with either specific antigen VIP (synthetic porcine, Cambridge Research Biochemicals 96% pure) or secretin (natural porcine, Karolinska Institute, 98% pure) at 10nmol antigen/ml diluted antibody. Positive controls consisted of fish gut tissue positive for VIP-like immunoreactivity and positive for secretin-like immunoreactivity. The methodology for the attachment of the antibody layers and the appropriate specificity controls are outlined diagrammatically in Fig. 7.2 and 7.3 respectively.

7.3 Results

Positive immunoreactivity for VIP-like material was found in all worms examined from day 4 to 11 postinfection. In all cases the immunoreactivity was confined to two locations ;

1. The distal cytoplasm, outer layer of the tegument,
2. the sub-tegumentary cells.

In some cases direct cytoplasmic continuity between these two sites could be demonstrated but even when this direct linkage was not apparent there was no doubt, from location and appearance, that the cells showing immunoreactivity were tegumentary cells.

Fig. 7.2 Attachment of antibodies

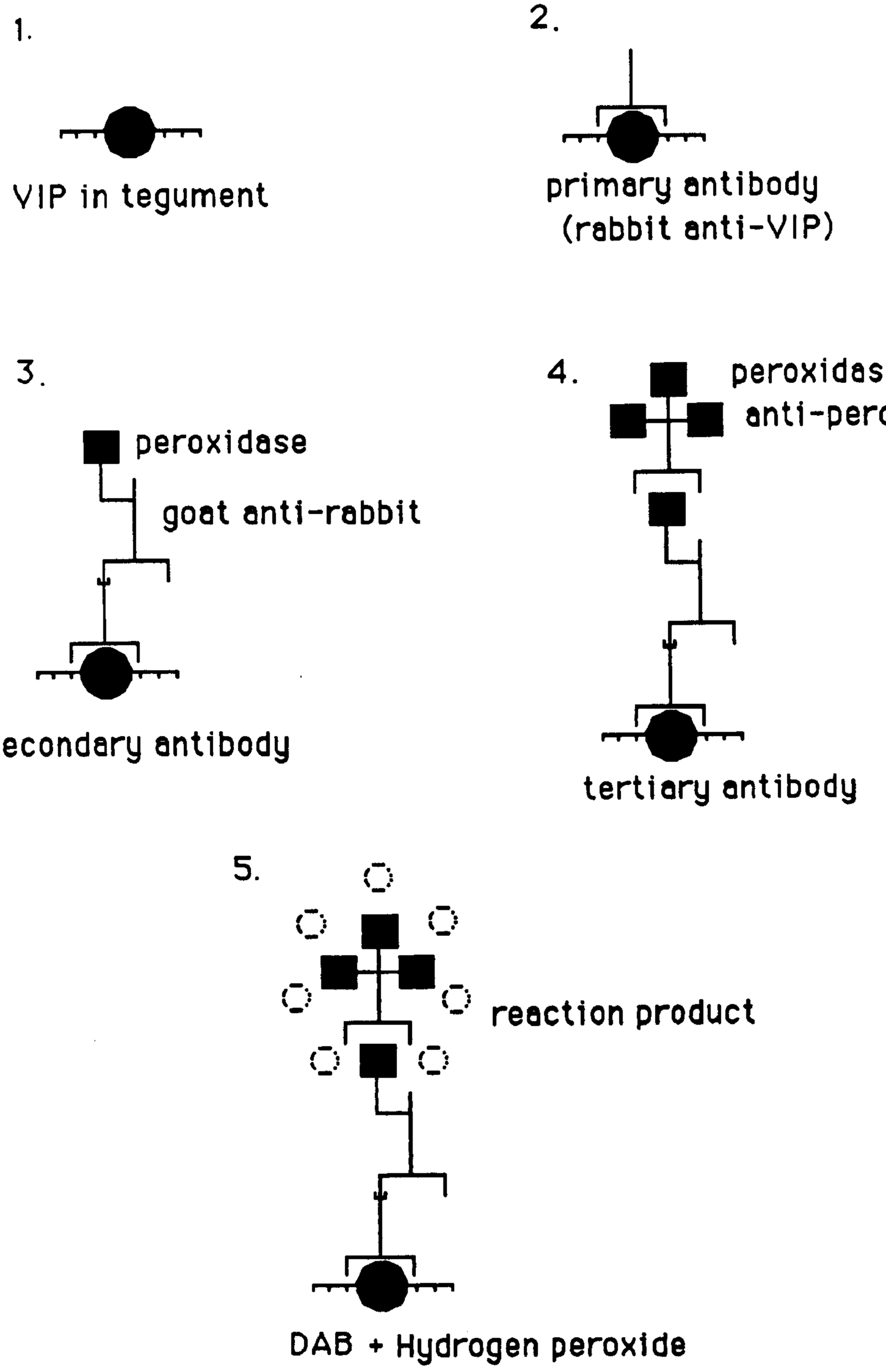
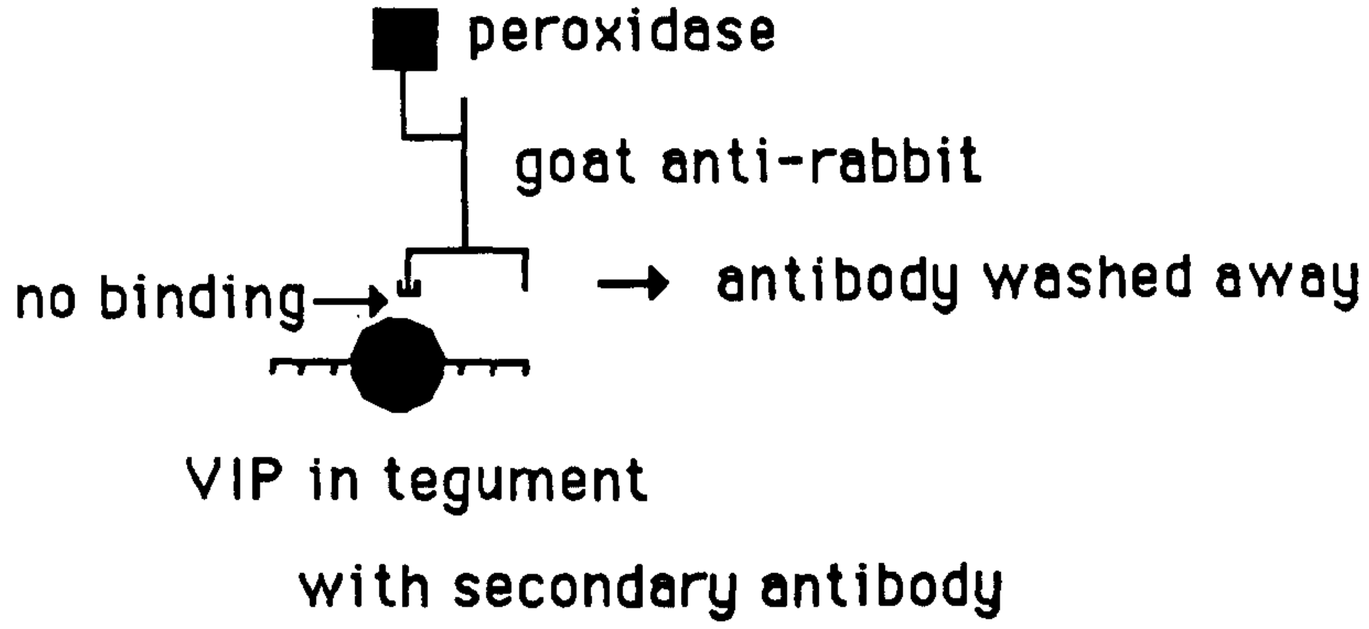
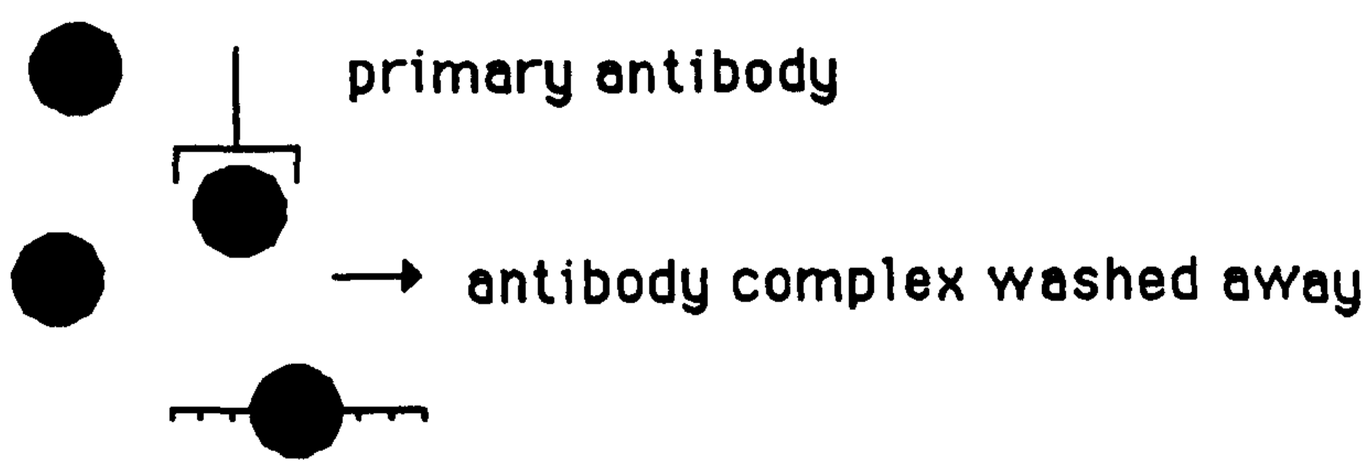


Fig. 7.3 Specificity controls

1. Omission of primary antibody



2. Preabsorption of primary antibody
with specific antigen



These two locations correspond to the known architecture of the digenean tegument which has been described as comprising an outer anucleate layer of cytoplasm connected by cytoplasmic strands to nucleated portions of cytoplasm, termed tegumentary cells (Smyth and Halton, 1983). The nature of this organisation will be discussed further in Section 7.4.

7.3.1 Immature worms

Day 4

Fig 7.4 (1) shows a section of a 4 day old worm. Immunoreactivity is confined mainly to the outer tegumental layer with some in a number of tegumentary cells. On the ventral surface immunoreactive tegumentary cells began posterior to the ventral sucker and continued in a posterior direction. On the dorsal surface the cells were in similar relative positions. The mean number of immunoreactive tegumentary cells on the ventral surface per linear 200 μm was 5, compared with 1.5 on the dorsal surface (see Table 7.1). No immunoreactive secretin-like material was identified.

Day 5

A relatively similar pattern of distribution with respect to the VIP-like immunoreactive tegumentary cells and tegument was found in these worms. In Fig 7.4 (2) a single immunoreactive cell of a 5 day old worm of *E. liei* is shown. This cell appears to have an immunoreactive extension directed towards the tegument of the ventral surface. The mean number of tegumentary cells on the ventral surface was 3.5 per 200 μm, on the dorsal surface 1.5. The

developing testes and ovary showed no reactivity and no secretin-like immunoreactivity was observed in these 5 day old worms.

Day6

In Fig 7.4 (3) and 7.4 (4) positive immunoreactivity is exhibited for both VIP and secretin-like material in cells of the tegument of 6 day old worms labelled with each of the two antisera. The VIP-like immunoreactive cells on the ventral surface (3.5 per 200 μm) were situated posterior to the ventral sucker and extended in a posterior direction while the immunoreactive tegumentary cells located on the dorsal surface (1.5 per 200 μm) showed no such distribution and were confined mid-way between the ventral sucker and the most posterior region of the worm. Not seen in the 4 and 5 day old worms, but present in these 6 day old worms was positive secretin-like immunoreactivity identified in the cells of the tegument. These immunoreactive cells were located exclusively on the ventral surface of the worms (2.5 per 200 μm) in a similar location to the VIP-like immunoreactive cells. In Fig 7.4 (4) the positive secretin-like staining cells appear to be tegumentary cells. Positive staining for secretin-like material is apparent in the cytoplasm and the cytoplasmic strands of these cells leading up to the outer tegument with some positive reactivity in the tegument itself. For both types of immunoreactivities, the cytoplasm of the tegumentary cells appeared to have abundant immunoreactive material whereas the nuclei of these cells had none. Although there was considerable spatial overlap in day 6 worms in the distribution of VIP immunoreactive and secretin immunoreactive sub-tegumentary cells, the techniques employed in this study did not enable a

Fig. 7.4 (1-5)

(1) Day 4 tegumental VIP-like immunoreactivity

Scale bar= 15 μm

(2) Day 5 tegumental cell VIP-like immunoreactivity

Scale bar- 5 μm

(3) Day 6 VIP-like immunoreactivity

Scale bar= 20 μm

(4) Day 6 tegumental cell secretin-like immunoreactivity

Scale bar= 12 μm

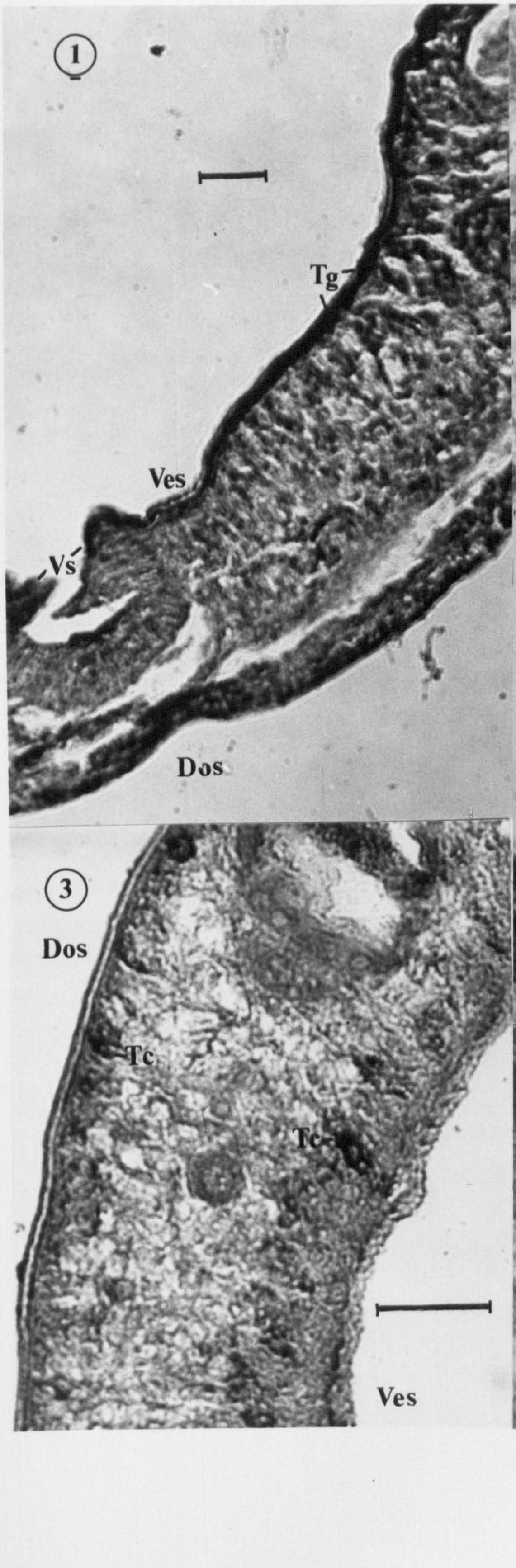
(5) Day 7 tegumental cell VIP-like immunoreactivity

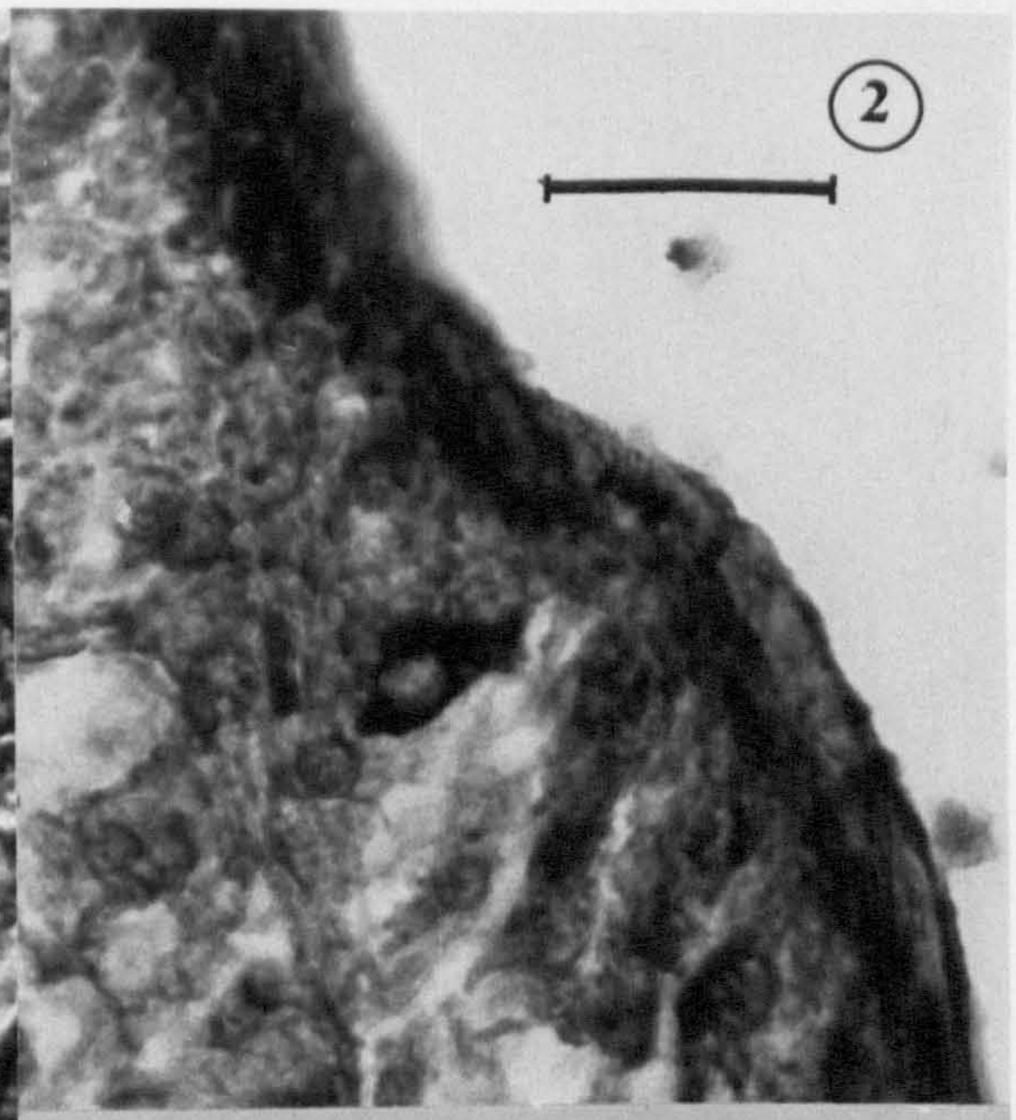
Scale bar= 12 μm

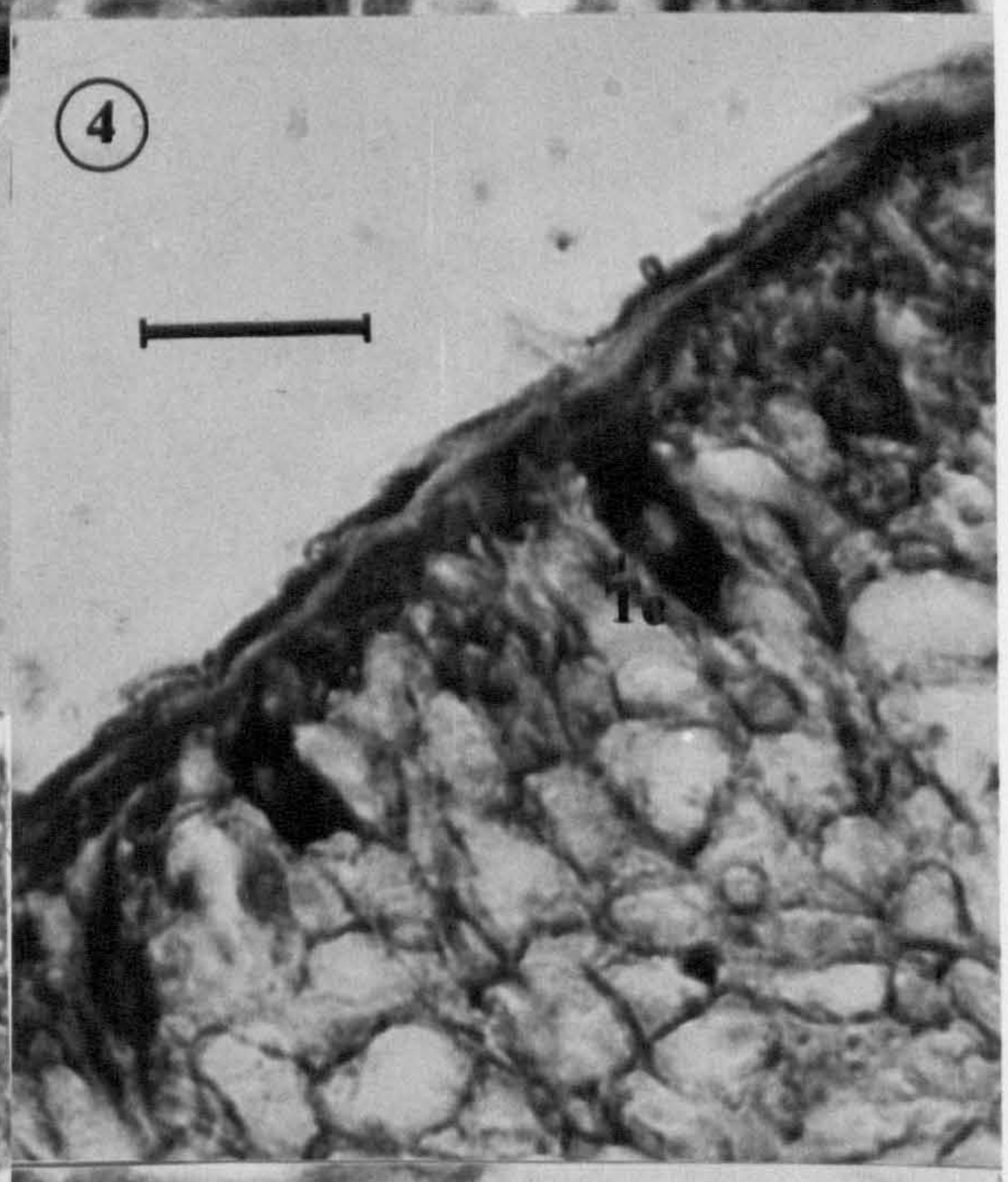
Key

Tg-tegument Ves-ventral surface Dos-dorsal surface

Os-oral sucker Vs-ventral sucker


Tg
Ves
Vs
Dos




Tc

Dos
Tc
Tc
Ves

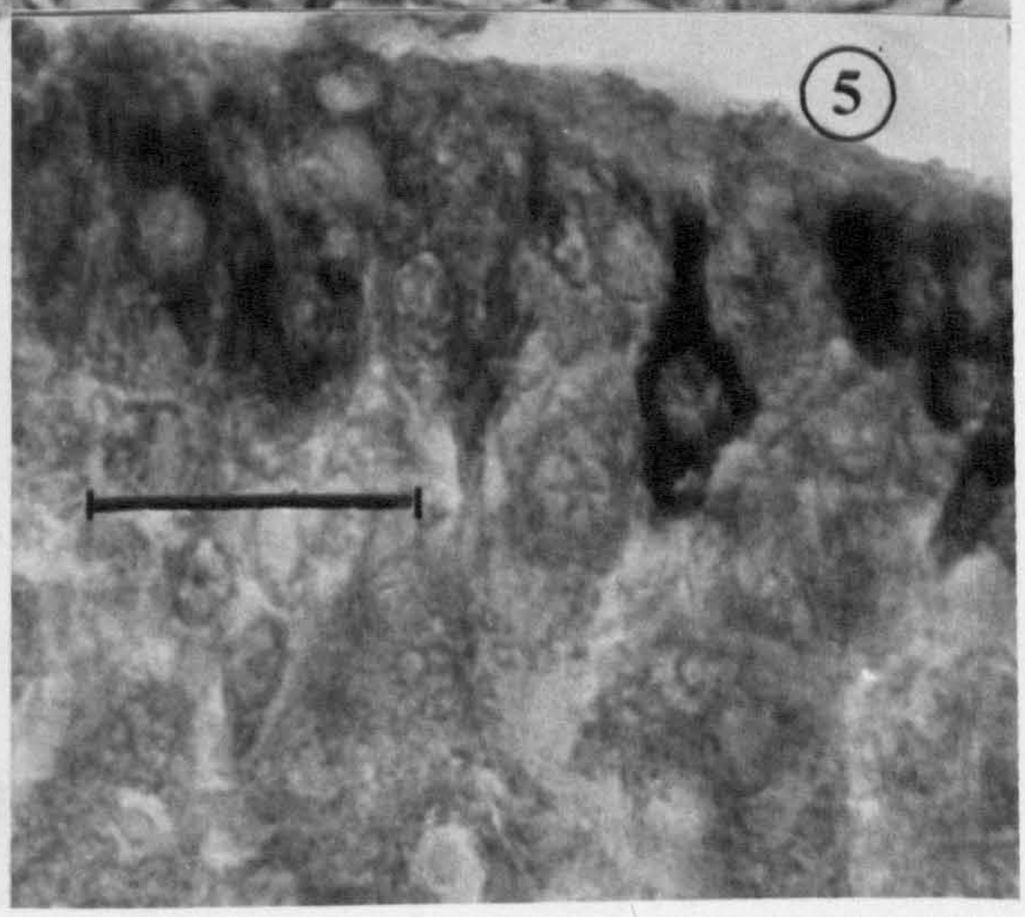


Fig. 7.4 (6-9)

(6) Day 8 tegumental cell VIP-like immunoreactivity

Scale bar= 16 μm

(7) Day 9 tegumental cell VIP-like immunoreactivity

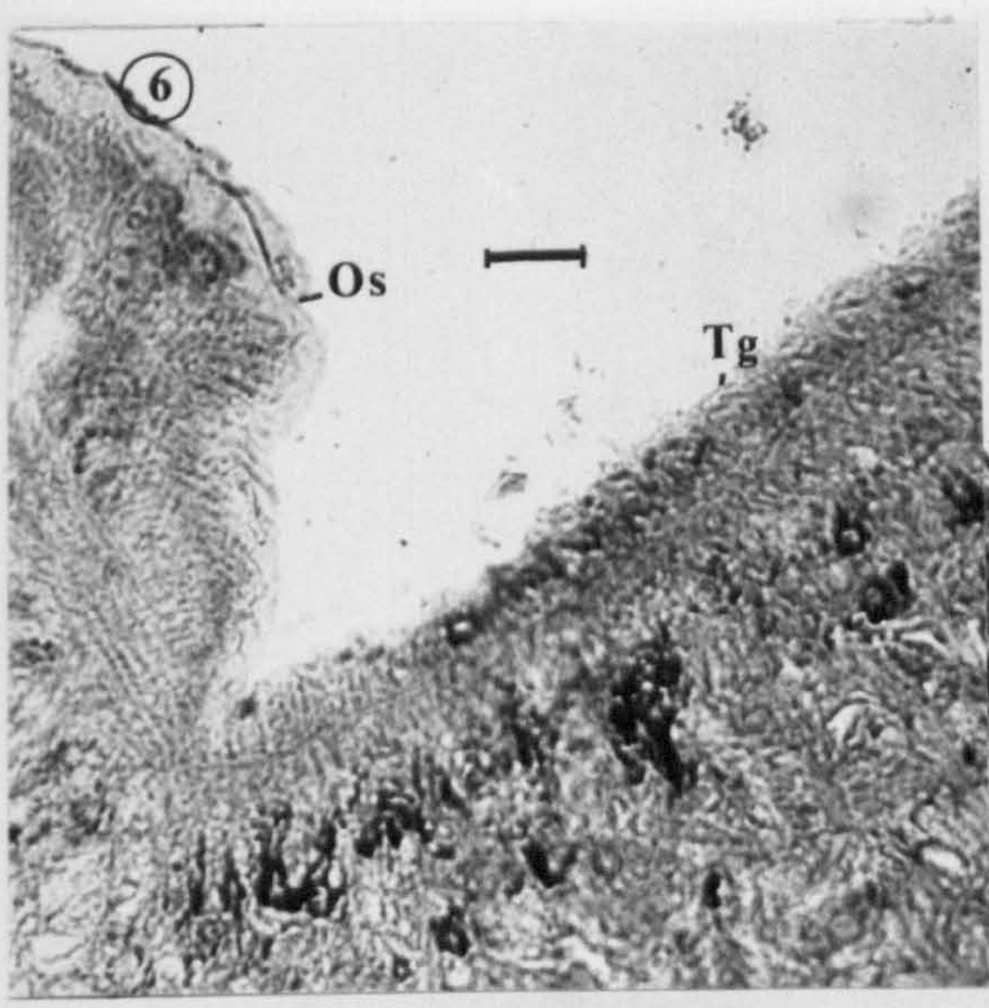
Scale bar= 20 μm

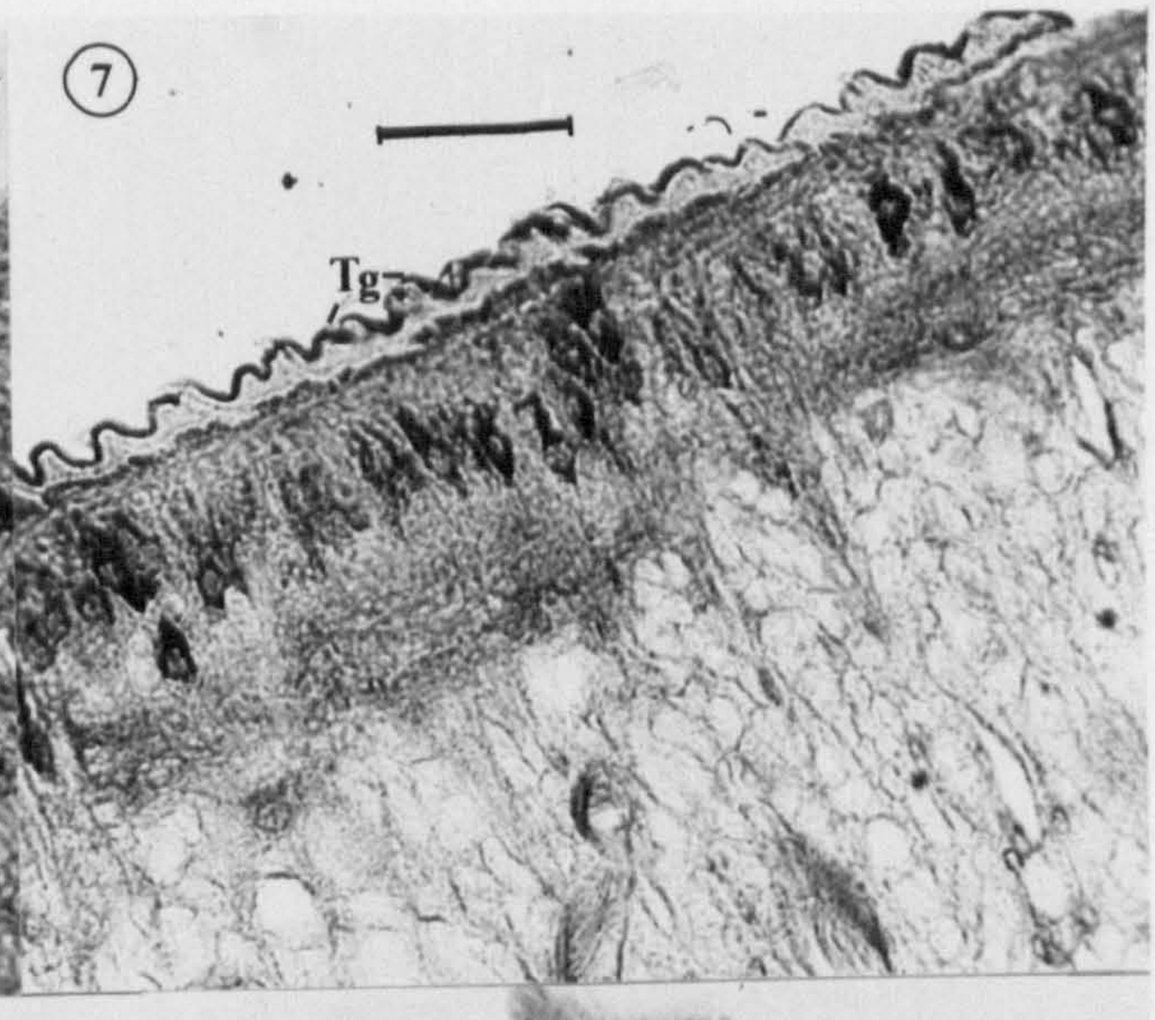
(8) Day 10 tegumental cell VIP-like immunoreactivity

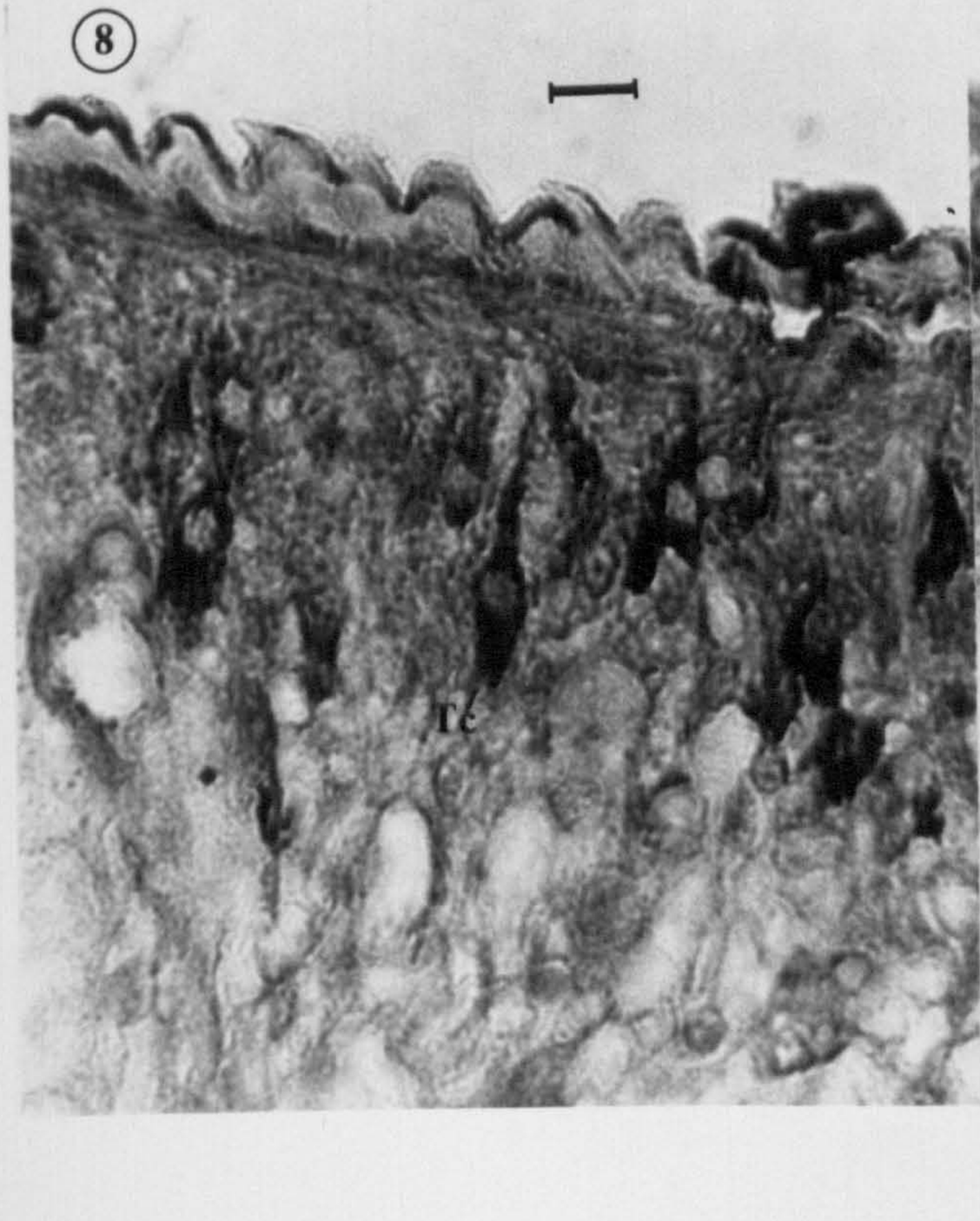
Scale bar= 10 μm

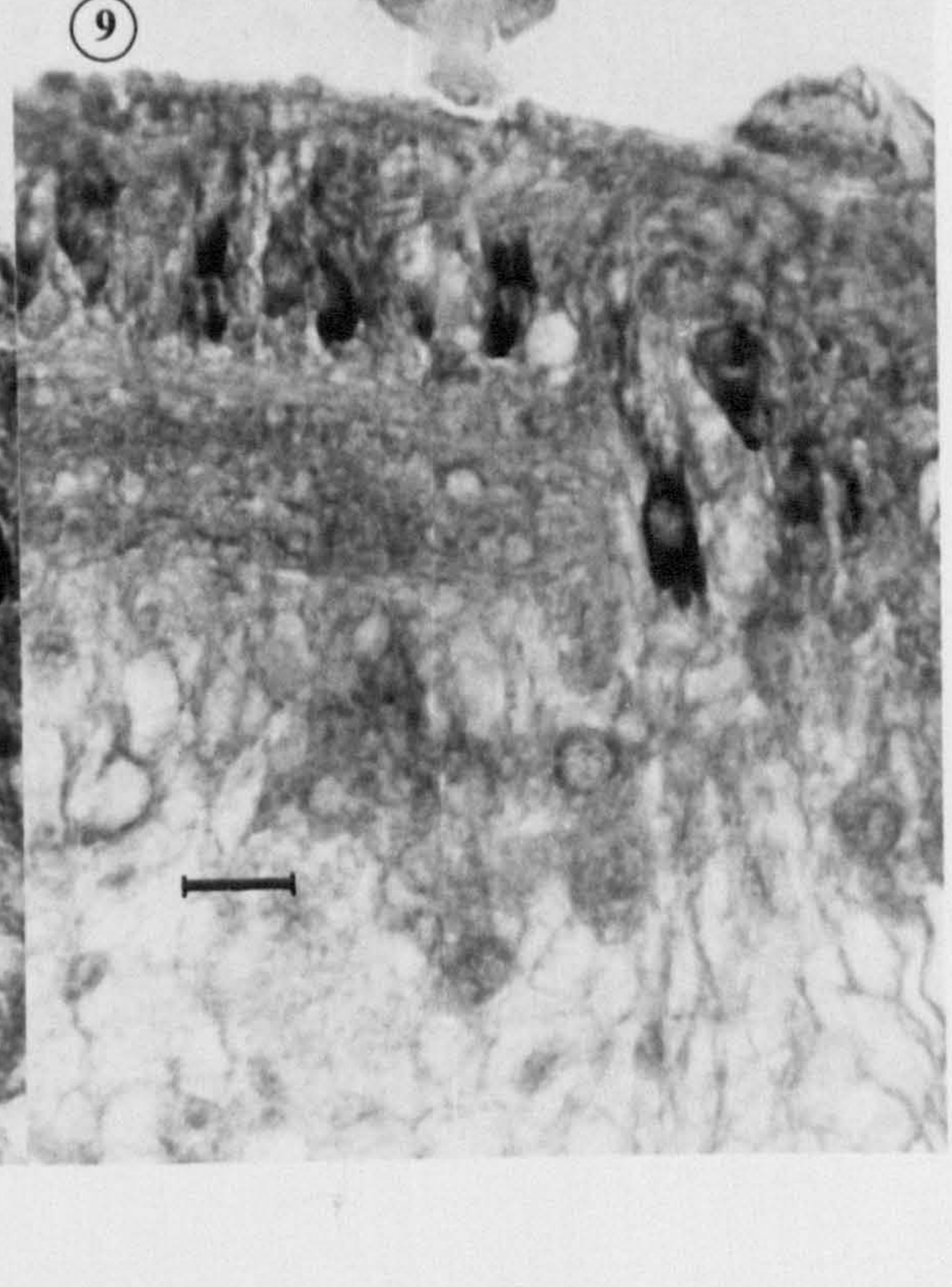
(9) Day 11 tegumental cell VIP-like immunoreactivity

Scale bar= 10 μm


6
Os
Tg


7
Tg


8
Te


9

decision to be made about whether both types of immunoreactivity could coexist in a single cell.

Day 7

Positive immunoreactivity for VIP-like material in tegumentary cells was identified (Fig. 7.4 (5)). In this instance, low levels of cell and tegumental immunoreactivity were seen on both the dorsal surface (1.5 per 200 μm) and ventral surfaces (2.5 per 200 μm). From Fig. 7.4 (5) it can be seen that the cells lie within the muscle layers of the wall of the developing worm. Immunoreactive cytoplasmic strands of these positive staining tegumentary cells can be seen being directed towards the outer tegument. Worms did not show any positive immunoreactivty when tested for secretin.

7.3.2 Sexually mature worms

Day 8

Worms showed positive immunoreactivity for VIP-like material in a number of cells of the tegument on the ventral surface but no immunoreactivity was seen on the dorsal surface. The immunoreactive tegumentary cells follow the distribution pattern described for the immature worms beginning posterior to the ventral sucker and continuing in a posterior direction (6.5 per 200 μm). Also in these worms immunoreactivity was seen in the region of the ventral surface between the ventral sucker and the oral sucker (Fig. 7.4 (6). No secretin-like immunoreactivities were observed.

Day 9

Immunoreactive VIP-like material was located in the tegumentary cells in these worms. Fig 7.4 (7) shows a group of immunoreactive tegumental cells along the ventral surface of a 9 day old worm. VIP-like immunoreactivity was only located in the cells of ventral surface posterior to the ventral sucker extending in a posterior position (9 per 200 μm). No cells of the dorsal tegument displayed any positive reactivities. No secretin-like immunoreactivity was observed.

Day 10

Fig. 7.4 (8) is a high power view of a group of tegumentary cells of a 10-day old worm showing positive immunoreactivity for VIP-like material. In these same worms immunoreactivity was confined to the cells of the ventral surface (9.5 per 200 μm) and followed a similar distribution to the 9 day old worms. No secretin-like immunoreactivity was observed.

Day 11

A similar immunoreactive distribution pattern to that of 9 and 10 day old worms was also observed in 11 day old worms with immunoreactivity absent from the dorsal surfaces. A high power view of immunoreactive tegumentary cells is shown in Fig. 7.4 (9). The linear measure of tegumentary cells was 8.5 per 200 μm . Secretin-like immunoreactivity was not observed in any of the 11 day old worms examined.

With respect to the controls utilized the primary antibody ommission controls gave negative results and the preabsorption

controls with both VIP and secretin also gave negative results. The sections of fish gut tissue used gave positive results.

Quantitative immunoreactive cell changes during development

Fig. 7.5 illustrates the changing density of VIP-like cells (mean number per 200 μm of tegument length, with attached standard error bars) on the ventral surface with developmental age of the worms. It is clear that there is a significant linear increase in density with age ($N=8$; $R=0.797$; $P<0.05$). Length and width measurements carried out on the immunoreactive cells at each developmental age involved measurement of the cell body only, excluding the cytoplasmic extensions of the cells. Figs. 7.6 and 7.7 plot the changing mean length and width of VIP-like immunoreactive cells with time (including the attached standard error bars). The mean length increases significantly with development ($N=8$; $R=0.782$: $P<0.05$) but the mean width does not ($N=8$; $R=0.619$; $P>0.05$).

TABLE 7.1 The mean lengths, widths and number of immunoreactive cells in the tegument of E. liei.

Days postinfection	Ventral surface			Dorsal surface		
	Mean length (microns)	Mean width (microns)	No. of cells/200 microns	Mean length (microns)	Mean width (microns)	No. of cells/200 microns
4	3.5 (+/-0.8)" n=3	2.5 (+/-0.0)^	5.0 (+/-1.0)*	2.5 (+/-0.0)*	2.5 (+/-0.0)*	1.5 (+/-0.5)*
5	3.7 (+/-1.2) n=2	3.7 (+/-1.2)	3.0 (+/-1.0)	2.5 (+/-0.0)	2.5 (+/-0.0)	1.5 (+/-0.5)
6	8.7 (+/-1.2) n=2	7.5 (+/-0.0)	3.5 (+/-0.5)	5.0 (+/-0.0)	6.2 (+/-1.2)	1.5 (+/-0.49)
6 (Secretin)	6.7 (+/-1.0) n=2	5.0 (+/-0.0)	2.5 (+/-0.5)	0.0 (+/-0.0)	0.0 (+/-0.0)	0.0 (+/-0.0)
7	6.2 (+/-1.2) n=2	5.0 (+/-0.0)	2.5 (+/-0.5)	6.2 (+/-1.2)	7.5 (+/-0.0)	1.5 (+/-0.5)
8	8.3 (+/-0.8) n=3	7.5 (+/-0.0)	6.5 (+/-0.5)	0.0 (+/-0.0)	0.0 (+/-0.0)	0.0 (+/-0.0)
9	9.4 (+/-1.2) n=4	6.2 (+/-1.2)	9.0 (+/-2.0)	0.0 (+/-0.0)	0.0 (+/-0.0)	0.0 (+/-0.0)
10	8.1 (+/-0.6) n=4	6.2 (+/-0.7)	9.5 (+/-0.5)	0.0 (+/-0.0)	0.0 (+/-0.0)	0.0 (+/-0.0)
11	8.7 (+/-0.7) n=4	6.2 (+/-0.7)	8.5 (+/-1.5)	0.0 (+/-0.0)	0.0 (+/-0.0)	0.0 (+/-0.0)

*n=2
^n as for ventral surface mean length
" Figures in brackets refer to standard errors

Fig. 7.5 Immunoreactive tegumentary cells per linear measure (data points include standard errors)

Fig. 7.6 The mean length of immunoreactive tegumentary cells (data points include standard errors)

Fig. 7.7 The mean width of immunoreactive tegumentary cells (data points include standard errors)

Fig. 7.5

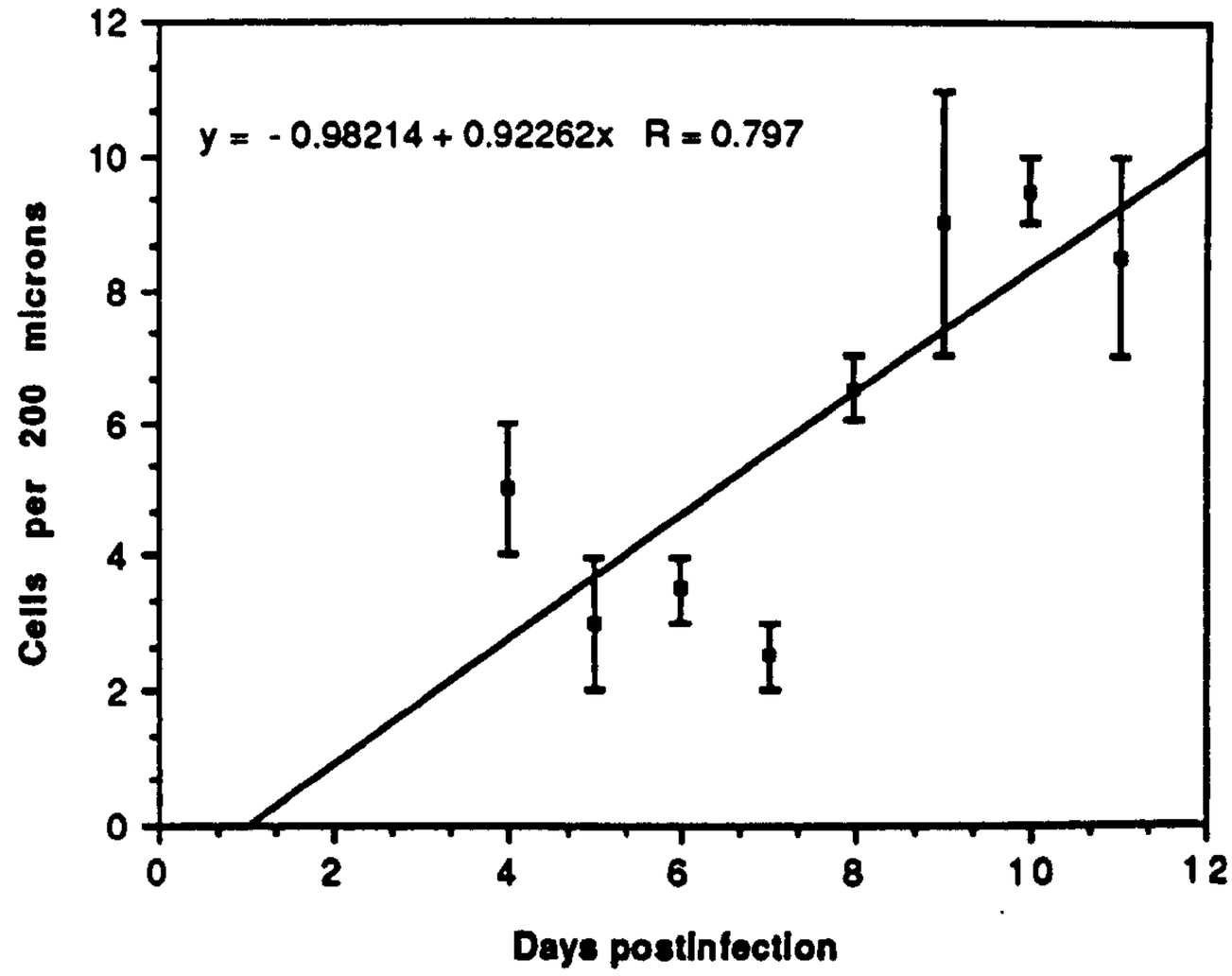


Fig. 7.6

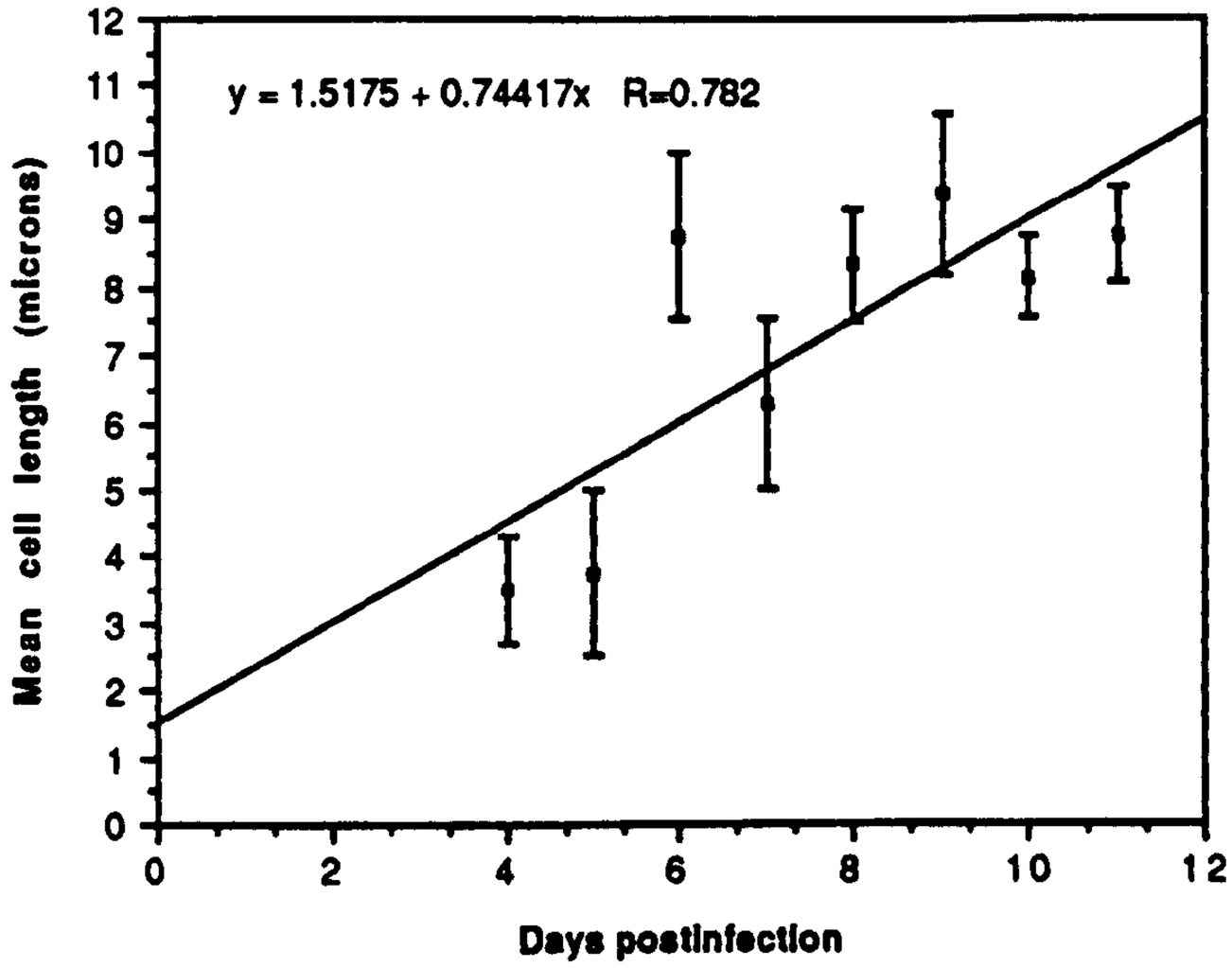
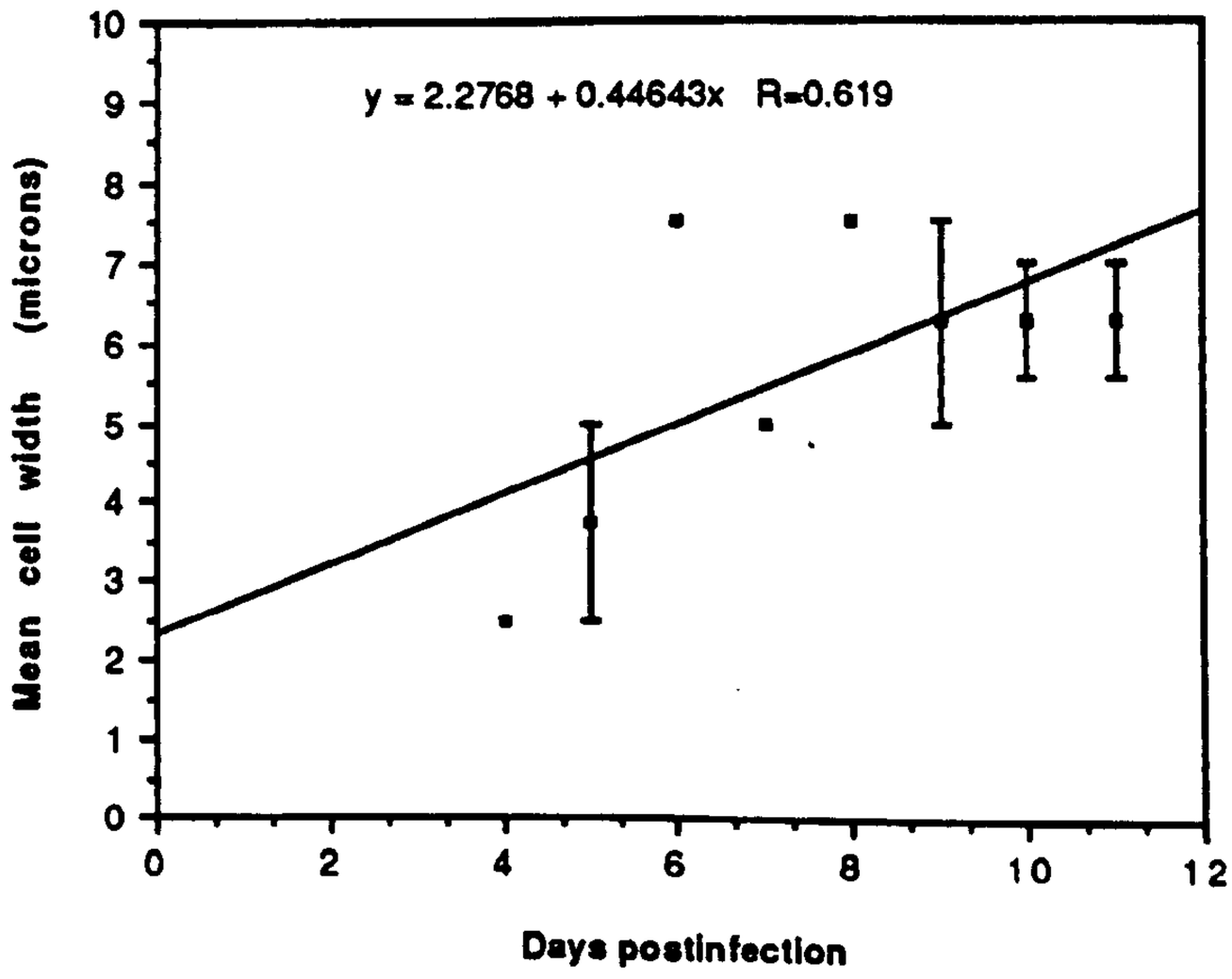


Fig. 7.7



7.4 Discussion

The immunoreactivities found in this study all relate to molecules immunologically related to vertebrate peptides localized in sub-tegumentary cells and the distal cytoplasm of the tegumentary syncytium of *E. liei*. This pattern of distribution highlights the potentially crucial role of the tegument in the synthesis, translocation, storage and secretion of macromolecules. Before the significance of the VIP and secretin findings are discussed it is appropriate to review what is known about these functional/structural aspects of the digenean tegument.

7.4.1 The nature of the tegument and associated synthetic functions

The tegumental surface of endoparasites forms an interface with the host environment and it would be therefore expected that major biochemical, physiological and immunological interactions take place at this interface. Threadgold (1963) demonstrated, for the first time, the living tegumental surface organisation of the digenean *Fasciola hepatica*. Subsequent ultrastructural work on *Fasciola* (Bjorkman and Thorsell, 1964 and Threadgold, 1967) has shown that the tegument of the adult worms is composed of two types of tegumentary cells which lie below the peripheral musculature and are connected to a distal syncytial layer by cytoplasmic connections. These cells were termed type 1 (T-1) and type 2 (T-2). T-1 cells were shown to be in the majority and contained numerous mitochondria and many small ovoid granules while the T-2 cells occurred only in groups of T-1 cells and

contained fewer mitochondria and many small biconcave disc-like granules. In contrast to the adult form of *F. hepatica*, the tegument of the newly excysted juvenile has only one type of tegumental cell (Bennett and Threadgold, 1973). This cell was found to be different from the adult tegumental cell types 1 and 2 and was therefore referred to as a type 0 (T-0) tegumental cell. Earlier work by Bennett and Threadgold, (1975) had demonstrated the importance of the addition of new tegumentary cells to pre-existing cells of the tegument at different time intervals during development. These workers looked at the changes in the tegument of juvenile worms of *Fasciola* from their excystment in the mouse gut and during their ontogenetic migration to the host bile duct. They established a link between migration from the stomach to the bile duct and development of the tegument. Firstly T-0 cells transformed to T-1 cells, this involved the T-0 cells that were already attached to the distal cytoplasm changing their secretory behaviour and beginning to produce new types of inclusions. Secondly, they noted that new cells (T-2) differentiated from embryonic cells in the parenchyma and made cytoplasmic connections with the distal cytoplasm. Both of the developing T-1 and T-2 cells were shown to produce cytoplasmic inclusions that as well as residing in the cytoplasm of the cell bodies passed into the distal cytoplasm. By the time the worms reached the liver at 3 weeks postinfection T-1 and T-2 cells were both present in the tegument and the granules of the original T-0 cells were found to be on the decline. At 6-weeks postinfection worms were present in the bile ducts of hosts, as the T-1 and T-2 cells were fully formed and the syncytium filled with granules

from both of the cells by the fusion of the cells with basal plasma membrane of the syncytium. Other morphological changes that occurred while the worms were present in the liver involved the formation of basal invaginations with associated vacuoles and an increase in the number of mitochondria. These workers indicated that these sequential developmental changes along with the appearance of secretory bodies in the tegument were a response to the changing demands of the host environment. Using *Fasciola* as a basic model of the organisation of the tegument of worms that inhabit the intestinal tract and its offshoots, it can be envisaged that the digenean tegument is a dynamic structure. It is a structure which has been described as being a metabolically active layer, morphologically specialised to function in the absorption of nutrients, the synthesis and secretion of materials, osmoregulation and excretion and having an indirect sensory role (Smyth and Halton, 1983).

A synthetic function for the digenean tegument is confirmed by the identification of numerous membrane bound inclusion bodies which originate in the tegumental cells and are transported through the cytoplasmic connections to the outer syncytium (Smyth and Halton, 1983). In some of the digeneans studied such as *Leucochloridiomorpha constantiae* (Harris, Cheng, Cali, 1974) *Cyathocotyle bushiensis* (Erasmus, 1967) and *Haplometra cylindracea* (Threadgold, 1968) ultrastructural observation points to the production of a single type of secretory body within a single type of tegumental cell. Slight variations of this fundamental

feature have provided further ultrastructural evidence for two types of inclusion bodies in adult worms both of which have been believed to have their origin in the same tegumentary cell. Examples of this pattern include *Schistosoma mansoni* (Hockley, 1973) and *Megalodiscus temperatus* (Bogitsh, 1968). A similar arrangement has been recorded in adult *Fasciola* as indicated earlier but in this worm each of the two distinct inclusion bodies is manufactured in the T-1 and T-2 tegumentary cells (Bennett and Threadgold, 1975). There is evidence that the secretory bodies of the tegument contain lytic enzymes as acid phosphatases have been demonstrated in both *F. hepatica* (Thorpe, 1968) and *S. mansoni* (Wheather and Wilson, 1976). Ultrastructural observations of the tegumentary surface has also revealed that the tegumentary cells display morphological features similar to those cells that are engaged in protein synthesis and export (Threadgold, 1963). They have large amounts of granular endoplasmic reticulum and mitochondria and Golgi apparatus that in *Haematoloechus medioplexus* have been found in the syncytium (Bogitsh, 1971).

Quantitative evidence for protein synthesis has been demonstrated for *F. hepatica* by Hanna and Threadgold (1976) which arose from stereological analysis of the adult worms surface. They used cyclohexamide, an inhibitor of cytoplasmic protein synthesis, to reduce production of the T1 sec retory bodies by the Golgi apparatus. Cyclohexamide was unable to prevent transport and discharge at the tegumental surface but dinitrophenol and iodoacetate were both found to block both synthesis and transport

of the secretions to the surface syncytium. Hanna (1980) has also confirmed that protein synthesis takes place in T1 cells of *Fasciola* and that the events are mediated by a GER-Golgi mechanism. Hanna (1976) has also demonstrated that the T-1 secretory bodies of *Fasciola* also contain carbohydrate. This mixture of evidence clearly supports the idea that these cells have synthetic capabilities. The release of the contents of these tegumental inclusions has been assumed to take place at the surface of the distal cytoplasm by exocytosis leading to the insertion of molecules into the glycocalyx (Hanna, 1980). The glycocalyx in *Fasciola* has been shown to be mainly composed of glycoproteins and glycolipids (Threadgold, 1976). Its exact role has not been defined but owing to its position and intimate contact with the outer environment it may be important in the protective, absorptive and immunological properties of the tegument (Smyth and Halton, 1983).

Thorndyke and Whitfield (1987) suggested that the VIP-like immunoreactive material identified in *E. liei* was being synthesised in a specialized sub-population of tegumentary cells and then transported up to the distal cytoplasm because of the nature of the association of the tegument and the positive staining they observed for VIP-like material in the outer tegumental layer. This theory is supported by the available knowledge on the nature of the digenean surface tegument and the cytological patterns of immunoreactivity found in the present study do nothing to diminish the likelihood that this is the case. On the whole, the tegumental cells of all parasitic platyhelminths including digeneans

are believed to be nucleated sections (cell bodies or perikarya) of an extensive probably unified multinucleate syncytium (Whitfield, 1979; Smyth and Halton, 1983). In a digenean such as *E. liei* this syncytium constitutes almost the entire surface of the worm and is divided into the outer surface of the worm (that is the nonnucleated distal cytoplasm) and numerous mononucleate tegumentary cells (Thorndyke and Whitfield, 1987). The tegumentary cells are confluent with the syncytium via cytoplasmic connections like those described in *Fasciola* (Threadgold, 1967; Bennett and Threadgold, 1975).

7.4.2 Age-dependent immunoreactive patterns in *E. liei*

A tegumental location of vertebrate-like peptides like that reported here and by Thorndyke and Whitfield (1987) in *E. liei*, has not been reported in any of the invertebrate systems investigated using antisera to known vertebrate peptides. A comparable example where the surface of the animal is utilized and demonstrates immunoreactivity to peptides is the work by Erspamer (1984) on amphibian skin which has been shown to synthesize large quantaties of bioactive peptides. These peptides, within frog skin in particular, have aroused considerable interest because they regularly have counterparts in mammalian tissues the gastrointestinal tract and the brain where they are represented by identical or analogous molecules (Erspamer, 1984)

Secretin-like immunoreactive material was only identified in 6 day old worms. Secretin is one of the chief stimulants of pancreatic

secretion relating to water, bicarbonate and pepsin in man and has been shown to inhibit gastric secretion of acid and motility of the gastrointestinal tract (Chey, Rhodes and Tai, 1978). There is no apparent previous documentation of the presence of secretin in invertebrate tissue although glucagon-like material has been demonstrated in insects (Tager, Markese, Kramer, Spiers and Childs, 1976).

Subjecting the day 6-8 time period to detailed consideration, it is interesting to note that there are distinct changes operating around this period in addition to the short-lived presence of secretin-like immunoreactivity on day 6. The presence of VIP-like tegumentary cell immunoreactivity from 4 days onwards is coupled with the increasing length of these cells. Between days 6 and 8 the number of VIP-like immunoreactive cells on the ventral surface decreases on day 7 but rises thereafter from day 8, reaching a plateau at day 9, 10 and 11. Interestingly, during this day 6-8 time period, VIP-like immunoreactivity ceases on the dorsal surface at day 7. Thereafter VIP immunoreactivity is confined to the ventral surface. This complex sequence of events relating to neuropeptides appears to have a temporal correlation with sexual maturity and reproduction since maturity is reached at 8 days.

There are many possible types of functional significance that could be ascribed to these changes in peptide synthesis and localization. For instance, it is conceivable that the short-lived production of secretin is in some way a developmental "triggering" event (or

associated with the timing of some other trigger). Day 6 is a period immediately before sexual activity begins in these digeneans, so the secretin "burst" might initiate any of the crucial physiological aspects of this activity. Secondly, if the secretin-like material is released from the tegument around day 6, it might play a direct role in stimulating aspects of inseminatory behaviour. In other words it could be operating as a reproductive pheromone influencing potential copulatory partners.

This last proposed role for secretin is intriguing since Riddell, Whitfield, Balogun and Thorndyke (1991), have demonstrated the presence of FMRFamide-like immunoreactive cells within the CNS and reproductive system of sexually mature *E. liei*. A solitary immunoreactive cell has been consistently found close to the terminal regions of the male reproductive tract, within the cirrus pouch. Considering the known neuromuscular function of FMRFamide (Price and Greenberg, 1980) it is envisaged that FMRFamide-like material in this cell may have effects on the ejaculatory musculature of the cirrus. Alternatively the cell might release its products into the seminal fluids with an effect being made on the musculature of the reproductive tract of a copulating partner after copulation.

In the only other study of neuropeptides in *Echinostoma*, Richard, Klein and Stoecke (1989) demonstrated substance P-like immunoreactivity in the worm's nervous system. Richard and her co-workers looked at 6 and 14-day old worms of *E. caproni*. In the

14 day old worms immunoreactive fibres and perikarya were observed in the central nervous system. Substance P immunoreactive fibres were observed in the central part of the ganglia, in the dorsal commissure and in the longitudinal nerve cords. In all these cases they noted that the nucleus was never labelled. In 6 day old worms similar observations were made, the only difference being that there was a marked overall increase in the substance P-immunoreactive nerve structures in the 14-day-old worms. In the 6-day old worms, substance P-like immunoreactivity was confined to the nervous system whereas in 14-day-old worms a strong substance P-like immunoreactivity occurred in the prostate gland lying within the cirrus pouch. They concluded that the immunoreactivity for substance P in the prostate gland was probably related to the age and sexual maturity of the worms. They explained that sexual maturity was reached at 7 days and they envisaged that the prostate cells begin to function at the same time, the absence of immunoreactivity prior to this time possibly being linked to sexual immaturity. This suggestion by Richard *et al.* mirrors that proposed above for *E. liei* and the short-lived production of a secretin-like neuropeptide.

A number of additional vertebrate-like neuropeptides have been localized in other parasitic worms such as *Hymenolepis diminuta* (pancreatic polypeptide PP, peptide tyrosine tyrosine PYY, peptide histidine isoleucine, gastrin-releasing peptide; Fairweather, *et al.*, 1988) and PP, PYY and substance P in *F. hepatica* (Magee, Fairweather, Johnston, Halton and Shaw, 1989) but in both cases

immunoreactivity was mainly associated with the nervous system indicating roles for these peptides as nervous and neuromuscular transmitters. In *Fasciola*, Magee *et al.* (1989) did comment on an additional role for PP in controlling the release of eggs from the ootype into the uterus and postulated that the native peptide FMRF-amide may be involved in influencing secretory releases from the Mehlis gland as well as controlling the movement of ova through the ootype. Maule, Halton, Johnston, Fairweather and Shaw (1989), demonstrated the immunological presence of PP, PYY and FMRFamide-like immunoreactivities throughout the central nervous system of *Diclidophora merlangi*. Association of these immunoreactive nerve fibres with the walls of the ootype, seminal vesicle and uterus led these workers to speculate that these peptides may be involved in egg development as well as having an integral role in neuronal functioning. They also noted the presence of VIP-like material in a population of nerve fibres that form part of the major longitudinal ventral nerve cord in the worm.

7.4.3 The pathophysiology of VIP

The position of VIP-like immunoreactivie material in the outer extremities of the tegument implies the possibility of exposure of epithelial cells of the small intestine to this parasite-produced material via loss of material from the glycocalyx. Endogenous mammalian VIP has a wide range of biological actions and although the physiological roles of this peptide are still unclear it is believed to be involved in a number of roles of which the following (see Said, 1981) are of particular relevance to the present study ;

1. Serving as a neurotransmitter both in the central and peripheral nervous system.

2. Mediating vasodilatory responses of the gastrointestinal circulation.

3. Relaxation of gut smooth muscle.

4. The regulation and stimulation of fluid and electrolyte secretion into the gut lumen.

The location of VIP-like immunoreactivity in nonneuronal cells appears to rule out a possible role as a neurotransmitter in this context in *E. liei*. The rest of the described roles have a significant bearing on the possible role of VIP in this gut-inhabiting worm.

Chapter 6 has described the pathology associated with sexually mature *E. liei* infections. The microhabitat utilization and aggregational behaviour of this worm (Chapter 5) through early development brings the worms into close proximity with one another and with the mucosal surface. With this and the nature of tegumental cells in mind, it is intriguing to speculate about the possible involvement of this parasite-produced peptide in host pathology. In a number of mammalian contexts, alterations in VIP levels and distribution are thought to be associated with gut pathology. In Crohn's disease, an inflammatory condition associated with villous pathology in humans, there is an increase in the number of VIP peptidergic fibres in the alimentary tract (Bishop, Polak and Bloom, 1981) and VIP has also been found to be associated with other clinical conditions. Plasma VIP has been found to increase markedly in the presence of hepatic failure in

dogs and humans (Ebeid, Escourrou, Murray and Fischer, 1978). It has been identified in tumours causing medullary thyroid carcinoma (MTC), where cell hyperplasia is evident. It has been speculated that VIP might play an indirect role in the watery diarrhoea syndrome often found in patients with MTC (O'Dorisio, 1978). Characteristic features of *E. liei* infections in mice described in Chapter 6 are villous atrophy, crypt hyperplasia, muscular hypertrophy and ballooning of the gut. These pathophysiological changes could be directly or indirectly generated by parasite-synthesized VIP-like material which could affect the villi of the jejunum and ileum, and underlying tissues. This syndrome of changes could be part of an adaptive strategy of the worms. The ballooning of the infected host seems likely to be due to release of liquids from gut tissues into the lumen. If this leakage contained organic materials utilized by *E. liei* the pathology could be seen as an alteration to enhance the feeding efficiency of the parasite.

If the suggested role of secreted VIP-like material by *E. liei* in parasite nutrition is indeed the case, it provides an interesting alternative method of mobilizing host nutrients extra-corporeally by gut-dwelling digeneans. The previously described method has been demonstrated in the gland cells of the strigeid *Holostephanus luhei* holdfast for instance. These cells have been found to be rich in proteins and RNA and secrete at the site of attachment, material containing hydrolytic enzymes that degrade the host tissue (Ohman, 1966). This is presumed to be extra-corporeal digestion as the host villi are denuded of their epithelium and disintegrated host tissue

is taken in by the oral sucker to the caeca where further digestion and absorption of the host tissue takes place (Ohman, 1966). Material leaking from the denuded villi are probably also directly absorbed by the strigeid's holdfast.

The persistence of VIP in the tegument of *E. liei* between days 4 and 11 would indicate that its presence is very important or essential. VIP material has been implicated in the host response to *Trichinella* infections in mice but in this instance it is believed that the peptide has arisen from host mast cells (Castro, 1982). As the tegument of digeneans is known to have an absorptive role, incorporation of host peptides onto the outer surface might be thought to be an alternative way of accounting for the VIP findings in this Chapter. The detailed localization of the immunoreactive material in *E. liei* suggests otherwise.

E. caproni has been found to release large amounts of antigen from its surface (Andresen, Simonsen, Andersen and Birch-Andersen, 1989) and also excretory-secretory products of *E. revolutum* have been found to have their origin at the worms surface (Andresen *et al.*, 1989). Bone (1982) has described primer pheromones associated with digeneans as causing physiological changes which later influence behaviour through hormonal changes. These studies imply that peptide release from the surface of gut-dwelling digeneans is a widespread phenomenon. The findings presented in this Chapter with respect to VIP-like and secretin-like polypeptides

have enabled detailed suggestions to be made about the nature and function of specific components of this polypeptide release.

CHAPTER 8

A COMPARISON OF THE REPRODUCTIVE SUCCESS OF SINGLE AND MULTIPLE WORM INFECTIONS OF *ECHINOSTOMA LIEI*

8.1 Introduction

The need to maximise reproductive success at low parasitic population densities, including the important limiting case of one parasite per host, would seem ecologically advantageous to parasitic worms whose host resource is often scarce and patchly distributed. Viable egg production from such infections, particularly those of single worms, would ensure the chance of some gene flow through to the next generation. It can be assumed that the hermaphrodite condition of most digeneans carries with it a potential built-in advantage in this context, in that it provides for the possibility of self-insemination. Hermaphroditism in digeneans however, also has a number of putative disadvantages including the fact that an unexpressed recessive mutant gene in one generation will appear in both female and male germ cells at the same time in the next generation (Smyth and Halton, 1983).

A number of experimental studies on digeneans have been carried out to assess the significance of the reproductive contributions of single worm infections. Fried (1962) found that isolated adults of *Philophthalmus hegeneri* stopped growing after 20 days in a chick's eye and never produced fertile eggs. Similarly, Howell and Bearup (1967) noted that single worm infections of *Philophthalmus burrili* were infertile and contained no recognizable seminal receptacle. In marked contrast to this, monometacercarial cyst infections involving *Zygocotyle lunata* (Bacha, 1966), *Philophthalmus megalurus* (Nollen, 1971), *Paragonimus*

westermani (Fan and Chang, 1970) and *Leucochloridiomorpha constantiae* (Fried and Harris, 1971) have all indicated that certain species of hermaphroditic digeneans are capable of producing eggs containing miracidia in conditions that preclude cross-insemination. This production presumably involves either self-fertilisation or parthenogenesis.

It has been suggested that cross-fertilisation is the most common form of reproductive process in adult digeneans (Smyth 1962; Smyth and Halton, 1983). Autoradiographic studies on multiple worm digenean adult infections have indicated that *Philophthalmus megalurus* (Nollen, 1968) and *Philophthalmus hegeneri* (Moseley and Nollen, 1973) will preferentially cross-inseminate. In multiple metacercarial cyst infections it may be assumed that conditions are available for cross-fertilisation and genetic variability can be generated by such crossing. In monometacercarial cyst infections however, where selfing is the only obvious means of achieving sexual reproduction of the type involving gamete fusion, there is presumably a significant genetic cost to be paid as self-fertilisation will result in a progressive reduction in genetic variability in the offspring.

Within the genus *Echinostoma*, Beaver (1937) has reported that single worms of *Echinostoma revolutum* are capable of producing viable eggs. Beaver observed this after removing 12 day old sexually immature worms from multiple infections in rats and

implanting them singly into the cloaca of pigeons. He found that these worms ultimately produced eggs which developed and hatched. This is an interesting result but one which, by itself, cannot demonstrate that selfing or parthenogenesis are occurring in this echinostome. The experimental design used by Beaver cannot exclude the possibility that the surgically transferred worms had already recieved sperm from cross-inseminating partners by day 12 of an infection. Lie and Owyang (1962) have more recently shown that monometacercarial cyst infections of *Echinostoma malayanum*, in both rats and hamsters, produced adult worms that expelled eggs which produced miracidia. Later studies involving monometacercarial cyst infections of *E. revolutum* conducted in chicks (Fried and Alenick, 1981) and hamsters (Fried, Huffman and Franco, 1988) and *E. caproni* in ICR mice (Fried and Sousa, 1990) and golden hamsters (Fried, Huffman and Weiss, 1990) have shown that these species can produce viable eggs under conditions which precluded cross-fertilisation. All of these authors have assumed that the mechanism for this viable egg production has been self-fertilisation.

The purposes of the present study were twofold. The first aim was to determine whether or not *E. liei* adults were capable of viable egg production in conditions that precluded cross-fertilisation. The second aim, assuming that such production is possible, was to investigate, over the course of multiple successive generations, the comparative reproductive success of worms which were always

present in mice at a density of one, and that of worms in multiple, potentially cross-fertilising infections. Several parameters were used to test the reproductive success of the single and multiple lines of adult worm infections. These included the mean metacercarial establishment success, the mean gut location of adult worms, mean dimensions of the adults, the mean number of uterine eggs per worm, the number of eggs produced per worm in 24 hours and the percentage egg viability assessed by hatching success. Further investigation of each infection line involved assessment of the ability of miracidia from the single and the multiple line infections to infect *Biomphalaria glabrata* and their subsequent ability to form infectious metacercarial cysts.

8.2 Materials and Methods

Section 8.2.1 and Section 8.2.2 provide descriptions of the main outlines of the experiments carried out over successive generations to establish a multiple worm cross-fertilising and a single worm selfing line of infection. Sections 8.2.3, 8.2.4 and 8.2.5, contain the protocol, adopted at each of the intermediary stages during each generation concerning adult worms and the infection of *B. glabrata* snails. The miracidial and cercarial infections of *B. glabrata* referred to in the following sections were carried out as documented in Section 2.6.1 and 2.6.2 respectively.

8.2.1 Multiple line infection

The initial multiple line was established from the previously maintained life cycle of *E. liei* in King's College laboratory (see Section 2.1). *B. glabrata* snails already harbouring mature metacercarial cysts of *E. liei* were used and cysts were dissected from the pericardial sacs of these snails. This initial batch of infective metacercarial cysts was obtained by pooling cysts from 20 infected snails from two separately maintained batches of stock infected snails. Five, 6-week old mice of *E. liei* were then each infected with 50 metacercarial cysts. This was the primary stage of the multiple line infection used to establish the first generation of worms (Generation 1, see Fig. 8.1, Step 1). Mice were then caged and fed as described in Section 2.7. Ten days postinfection the mice were necropsied and eggs teased from the uteri of randomly selected worms from all five mice (Random digit tables, Campbell, 1979), mixed and pooled as one sample (Step 2) and then incubated (see Section 2.6.1). After incubation and hatching the released miracidia were used to infect between 25 and 50 snails, 2 miracidia being used to infect individual snails (Step 3). Successfully infected snails which harboured and shed cercariae, were then used to infect uninfected snails with metacercariae. The cercariae shed from infected snails were allowed to mix so as to provide a mixed population of cercariae from a minimum of 20 snails (Step 4). Uninfected snails were then exposed to these cercariae which resulted in snails a (minimum of 15) containing a mixed metacercarial cyst population obtained from cercariae arising from

a number of different snails (Step 5). From this population of mixed metacercarial cysts the multiple line infection protocol was repeated to produce the second generation. Metacercarial cysts from the mixed stock were used to infect 5, 6 week-old mice with 50 cysts each (Step 6). In all, one complete generation of parasite development (that is from mouse infection to mouse infection in the next generation) took approximately 70 days.

8.2.2 Single line infection

To establish the first generation of this infection protocol, 15, 6 week old mice were each infected with 1 metacercarial cyst of *E. liei* (see Fig.8.2 Step 1). As in the multiple line infection protocol, these initial cysts were obtained from stock life cycle *B. glabrata* snails, harbouring mature metacercarial cysts of *E. liei* . Ten days postinfection the 15 mice were necropsied and eggs teased from randomly selected worms in the mice that had become infected. Eggs were not pooled (Step 2). Egg batches from individual worms were incubated separately and pairs of miracidia (each from the same egg batch) were used to infect uninfected snails (Step 3). Successfully infected snails that shed cercariae were then used to provide non-pooled groups of infective cercariae (Step 4). Uninfected snails were infected with metacercariae by exposure to groups of cercariae each derived from a single snail (Step 5). To establish the next generation of infected mice, fifteen 6-week old mice were each infected with a single metacercarial cyst from those in a single infected snail (Step 6). These procedures in the

Fig. 8.1 Establishment of multiple line infection

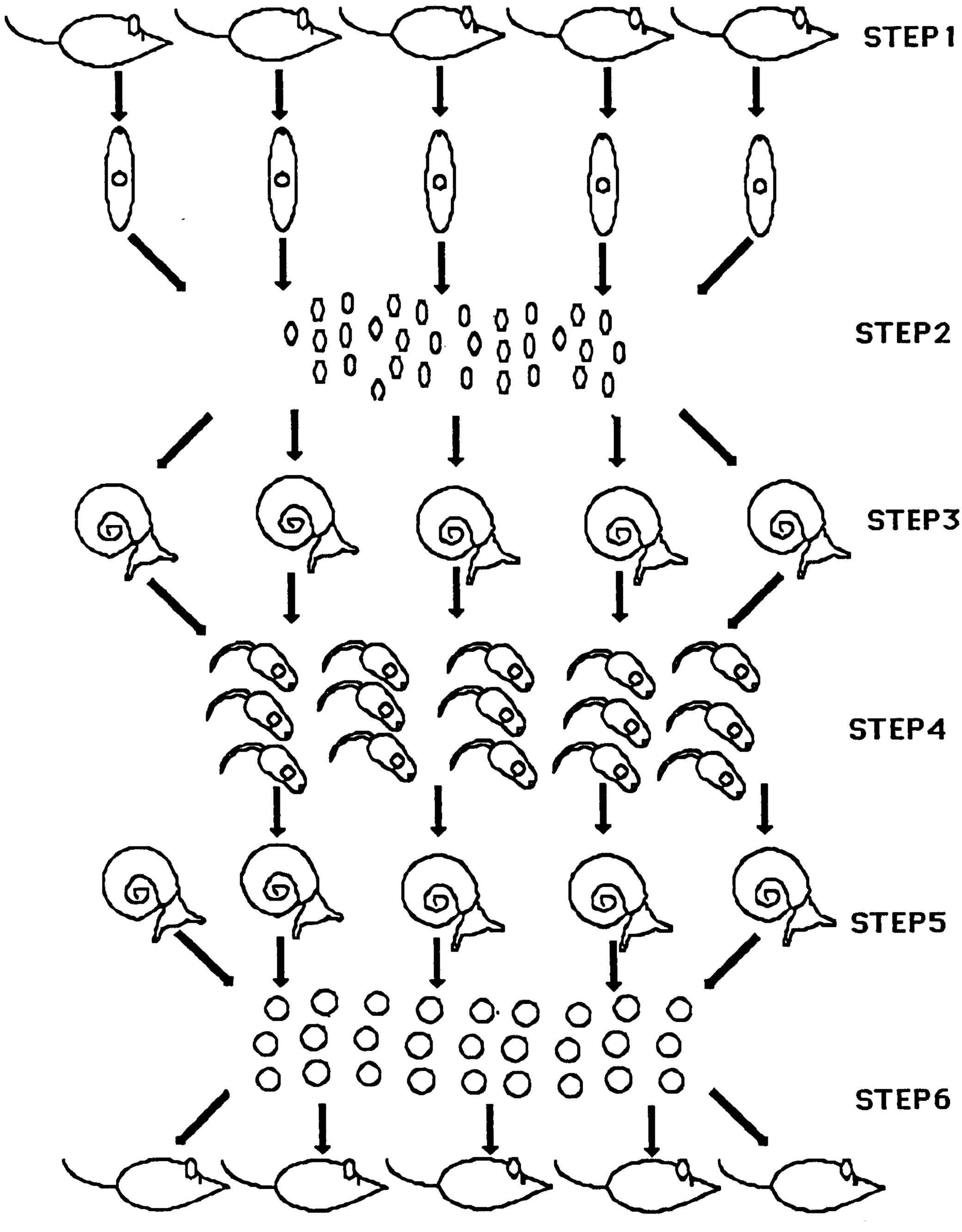
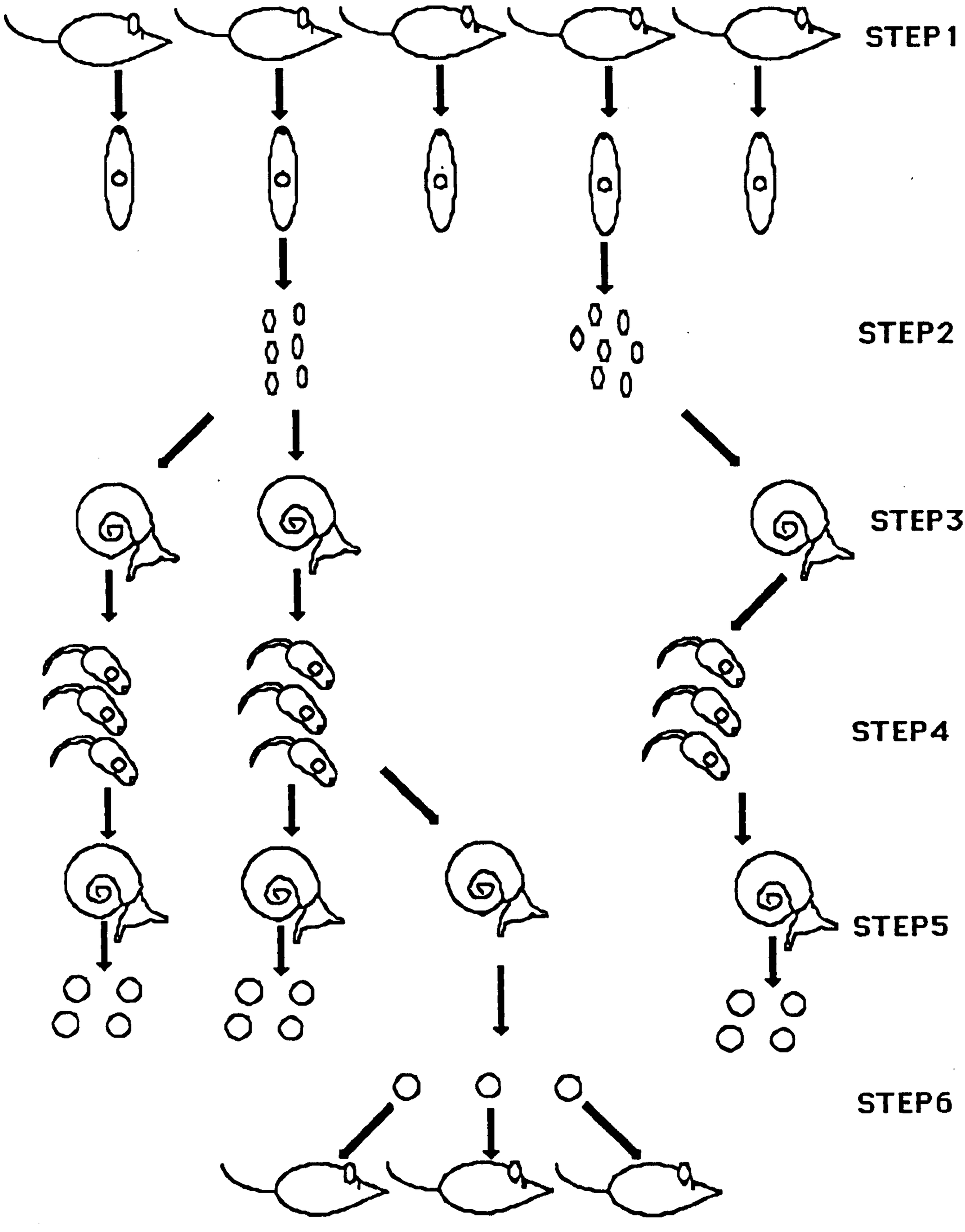


Fig. 8.2 Establishment of single line infection



protocol ensured that genetic mixing from egg production through larval development to cercarial production was kept to an absolute minimum, that is the genetic homogeneity of each successive population of adult worms was maximized.

8.2.3 Faecal analysis

All infected mice were placed in bottomless cages with a lower resevoir 24 hours before necropsy (at 10 days postinfection) to carry out faecal analysis as described in Section 2.7.1. Two weighed sub-samples were taken from each 24 hour faecal sample and the mean egg output in 24 hours was calculated (see Section 2.7.2). Mice were then necropsied.

8.2.4 Necropsy

At necropsy the small intestine was opened with a longitudinal incision and divided into five sections. In the multiple line infection the position of the worms in each of the 5 mice infected with 50 metacercarial cysts was first established by calculating the percentage position of the worms in the small intestine as described in Section 5.2.2. All worms were then removed from the host mucosa and counted. Randomly selected worms had eggs teased from their uteri which were pooled and incubated (for production of the next generation). Other randomly selected worms from this collection had the number of eggs contained in their uteri recorded. A number of worms from each of the infections had their lengths and widths measured after being fixed in Berland's (see

Appendix 1) solution for 15 seconds and then placed in 70% alcohol. In the single line infection, randomly selected worms were measured after Berland's fixation while the remaining worms had eggs removed from their uteri. These eggs were counted and then incubated.

8.2.5 Snail infection

In both single and multiple infection lines after incubation of eggs, hatching was stimulated as described in Section 2.5.1 and the percentage of hatched eggs determined in order to assess egg viability. After snail infection in both protocols the percentage of snails which had become infected by exposure to 2 miracidia was assessed 35 days after miracidial exposure.

8.2.6 Statistical analysis

A variety of quantitative parameters of the successive worm generations and infection processes were analysed statistically. Commonly, the mean values of parameters such as percentage worm gut position, dimensions of adults, number of uterine eggs per worm and the number of eggs produced per worm in 24 hours were compared between populations using the Student's t-test. Where the raw data was in the form of percentages it was first transformed using the arcsin transformation as recommended by Schelfer (1979). With respect to the number of eggs expelled in 24 hours and the number of uterine eggs, where the data took the

form of counts, the logarithmic transformation as recommended by Elliot (1983) was applied.

8.3 Results

These long-term, multigeneration experiments consisted of a period of continual host and parasite monitoring of at least 420 days. Within this extensive time frame a wide variety of parasite parameters were measured and collated. Given this time scale and complexity, it is an inevitably difficult matter to crystallize the principle patterns of the findings. To summarize these, the single and multiple line infections each persisted without break until Generation 4. During the course of this generation however, the single worm infection line suffered serious deleterious changes which prevented its continuance into Generation 5, the multiple line however persisted until Generation 6 when it was deliberately brought to an end. The data relating to the monitoring of parasites in the two lines through 4 and 6 generations respectively are summarized in Tables 8.1, 8.2, 8.3, 8.4 and 8.5. These show mean values in each line for the variables, establishment success, gut position, worm dimensions, number of eggs expelled per worm in 24 hours, the number of uterine eggs and the percentage egg viability. Figs 8.3, 8.4, 8.5, 8.6, 8,7, 8.8, 8.10 display graphically the changing mean values of these same variables throughout the multi-generation course of the two infection lines with attached standard error bars and Fig. 8.11 shows the percentage miracidial

infection success. The manner in which each of these epidemiologically significant variables altered throughout the generations of the experiment are now described in turn.

8.3.1 Establishment success

Establishment success (Fig. 8.3) of worms in both the multiple and single line infections showed much variability from generation to generation. Extreme mean values (both from the single line) were 33% to 87%. Despite this variability there was no clear indication that there was a marked difference in establishment success between the multiple and single lines. The mean value for the first four generations for the two lines were 62.2% and 66.7% respectively.

8.3.2 Gut positions

From Fig. 8.4 it is evident that gut location in the mouse intestine is less variable between generations in the single line infections in comparison with the multiple line infections over 4 generations. For these generations the single line worms appear always to inhabit a more posterior mean position along the gut (between 79% and 82%) compared with the mean positions of the worms in the multiple line which range from 66% to 77%. When statistically compared, these mean positions are significantly different at each generation (Generation 1, $t=10.02$, D.F.=18, $P<0.001$; Generation 2, $t=-3.12$, D.F.=6, $P<0.05$; Generation 3, $t=10.0$,D.F.=16,$P<0.001$; Generation 4, $t=-11.4$,D.F.=21,$P<0.001$).

TABLE 8.1 Summarised results of worms recovered from the single and mutiple line from the first and second generation

	Mean establishment (%)	Mean worm position (%)	Mean length (mm)	Mean width (mm)	Mean no.of eggs in 24 hrs	Mean no.of eggs per worm in 24 hrs	Mean no of in-utero eggs
GENERATION 1							
Multiple line	64.8 (+/-2.99)* n=5	69.9 (+/-0.46) n=162	5.57 (+/-0.18) n=46	1.21 (+/-0.12) n=46	5953.4 (+/-1024.6)	183.9 (+/-25.8)	96.7 (+/-14.1) n=15
Single line	66.7 (+/-0.13) n=15	79.3 (+/-0.94) n=10	4.07 (+/-0.02) n=10	1.26 (+/-0.05) n=10	556.4 (+/-58.8)	556.4 (+/-58.8)	265.8 (+/-43.7) n=5
GENERATION 2							
Multiple line	50.8 (+/-6.84) n=5	76.6 (+/-0.65) n=127	5.02 (+/-0.05) n=54	1.18 (+/-0.02) n=54	4697.8 (+/-901.5)	218.7 (+/-35.7)	189.7 (+/-21.4) n=15
Single line	33.3 (+/-0.13) n=15	81.8 (+/-1.48) n=5	4.29 (+/-0.24) n=5	1.35 (+/-0.05) n=5	637.5 (+/-65.4)	637.5 (+/-65.4)	297 (+/-8.98) n=2

*Figures in brackets refer to standard error of mean values

TABLE 8.2 Summarised results of worms recovered from the single and multiple line from the third and fourth generation

	Mean establishment (%)	Mean worm position (%)	Mean length (mm)	Mean width (mm)	Mean no.of eggs in 24 hrs	Mean no. of eggs per worm in 24 hrs	Mean no. of in-utero eggs
GENERATION 3							
Multiple line	54.8 (+/-1.96)* n=5	68.7 (+/-0.55) n=137	4.30 (+/-0.05) n=53	1.12 (+/-0.01) n=53	3933.5 (+/-709.5)	146.7 (+/-28.7)	216.1 (+/-23.03) n=15
Single line	80.0 (+/-0.11) n=15	80.5 (+/-1.48) n=12	4.57 (+/-0.11) n=12	1.29 (+/-0.06) n=12	564.2 (+/-54.3)	564.2 (+/-54.3)	210.8 (+/-52.7) n=4
GENERATION 4							
Multiple line	78.4 (+/-4.14) n=5	65.7 (+/-0.54) n=196	5.67 (+/-0.07) n=81	1.24 (+/-0.02) n=81	5799.2 (+/-921.2)	148.2 (+/-15.63)	125.1 (+/-8.71) n=15
Single line	86.7 (+/-0.09) n=15	80.4 (+/-1.03) n=13	4.45 (+/-0.12) n=13	1.12 (+/-0.03) n=13	433.3 (+/-29.5)	433.3 (+/-29.5)	161.1 (+/-17.65) n=7

*Figures in brackets refer to standard error of mean values

TABLE 8.3 Summarised results of worms recovered from the multiple line of the fifth and sixth generations

	Mean establishment (%)	Mean worm position	Mean length (mm)	Mean width (mm)	Mean no. of eggs in 24 hrs	Mean no. of eggs per worm in 24 hrs	Mean no. of in-utero eggs
GENERATION 5							
Multiple line	80 (+/-3.08)* n=5	64.5 (+/-0.67) n=200	3.84 (+/-0.04) n=78	1.13 (+/-0.01) n=78	8192.6 (+/-1027.8)	208.9 (+/-27.3)	201.5 (+/-13.4) n=15
GENERATION 6							
Multiple line	56.8 (+/-5.46) n=5	81.59 (+/-0.45) n=142	5.51 (+/-0.10) n=75	1.14 (+/-0.01) n=75	5235.3 (+/-1093.4)	190 (+/-37.61)	152.5 (+/-8.26) n=15

*Figures in brackets refer to standard error of mean values

TABLE 8.4 The percentage of hatched miracidia and infected snails at successive generations.

	Multiple line		Single line	
Generation	Mean % of hatched miracidia	No. of infected snails (%)	Mean % of hatched miracidia	No. of infected snails (%)
1	84.3 (+/-5.68)	34	79.3 (+/-4.34)	29.7
2	80.6 (+/-3.64)	21	92.2 (+/-2.95)	34.6
3	87.4 (+/-2.87)	20	95.6 (+/-1.92)	27.2
4	94.6 (+/-1.11)	10	3.79 (+/-0.78)	0.00
5	88.6 (+/-1.98)	26		
6	88.8 (+/-2.07)	26		

TABLE 8.5 Summary data of multiple infection and single infection using cysts derived from the fourth generation (single line)

	Establishment (%)	Mean worm position (%)	Mean length (mm)	Mean width (mm)	Mean no. of eggs in 24 hrs	No. of eggs per worm in 24 hrs	Mean no. of in-utero eggs
Mouse A	74	63.5 (+/-0.54)* n=37	3.75 (+/-0.08) n=7	0.92 (+/-0.02) n=7	1709.2 (+/-80.8)	46.2	72.9 (+/-13.1) n=14
Mouse B	62	33.3 (+/-0.70) n=31	3.63 (+/-0.04) n=12	0.94 (+/-0.03) n=12	1122.05 (+/-110.3)	36.2	67.3 (+/-12.2) n=14
Single infection	50^ (+/-0.17) n=10	80.7 (+/-1.52) n=5	3.97 (+/-0.01) n=5	1.11 (+/-0.04) n=5	394 (+/-85.1)	394^ (+/-85.1)	151 (+/-20.8) n=3

*Figures in brackets refer to standard error of mean values
^ Mean values

8.3.3 Worm dimensions

Fig. 8.5 and 8.6 respectively reveal that on the whole worms from multiple infections were greater in length in three out of the first four generations than worms from single infections but smaller in their widths. Single worms demonstrated comparable lengths throughout the four generations while the multiple worms were more variable. Less variability was seen in the widths of multiple and single worms but the single worms at Generation 4 were smaller than those in the previous generation. At Generation 1 analysis revealed a very significant difference in the mean length of worms (multiple line 5.57mm, single line 4.07mm, $t=5.07$, $D.F.=24$, $P<0.001$), but no significant difference in the width (multiple line 1.21mm, single line 1.26mm, $t=-0.361$, $D.F.=49$, $P>0.05$). At Generation 2 a significant difference was seen in both length (multiple line, 5.02mm; single line, 4.29mm; $t=2.9$,$D.F.=5$,$P<0.05$) and width (multiple line 1.18mm, single line 1.35mm; $t=-2.98$,$D.F.=P<0.05$). Between-line significant differences in both lengths and widths were found in Generation 3 (multiple line length 4.30 mm, single line, 4.57mm; $t=2.23$,$D.F=16$, $P<0.05$; multiple line width 1.12, single line, 1.29; $t=-2.82$,$D.F.=13$ $P<0.05$) and Generation 4 (multiple line length, 5.67mm; single line, 4.45mm; $t=6.81$, $D.F.=17$,$P<0.001$; multiple line width, 1.24mm; single line 1.12 mm ($t=3.01$,$D.F.=28$ $P<0.01$)).

Fig. 8.3 Mean percentage establishment success at successive generations (data points include standard errors)

Fig. 8.4 Mean positions of worms at successive generations (data points include standard errors)

Fig. 8.3

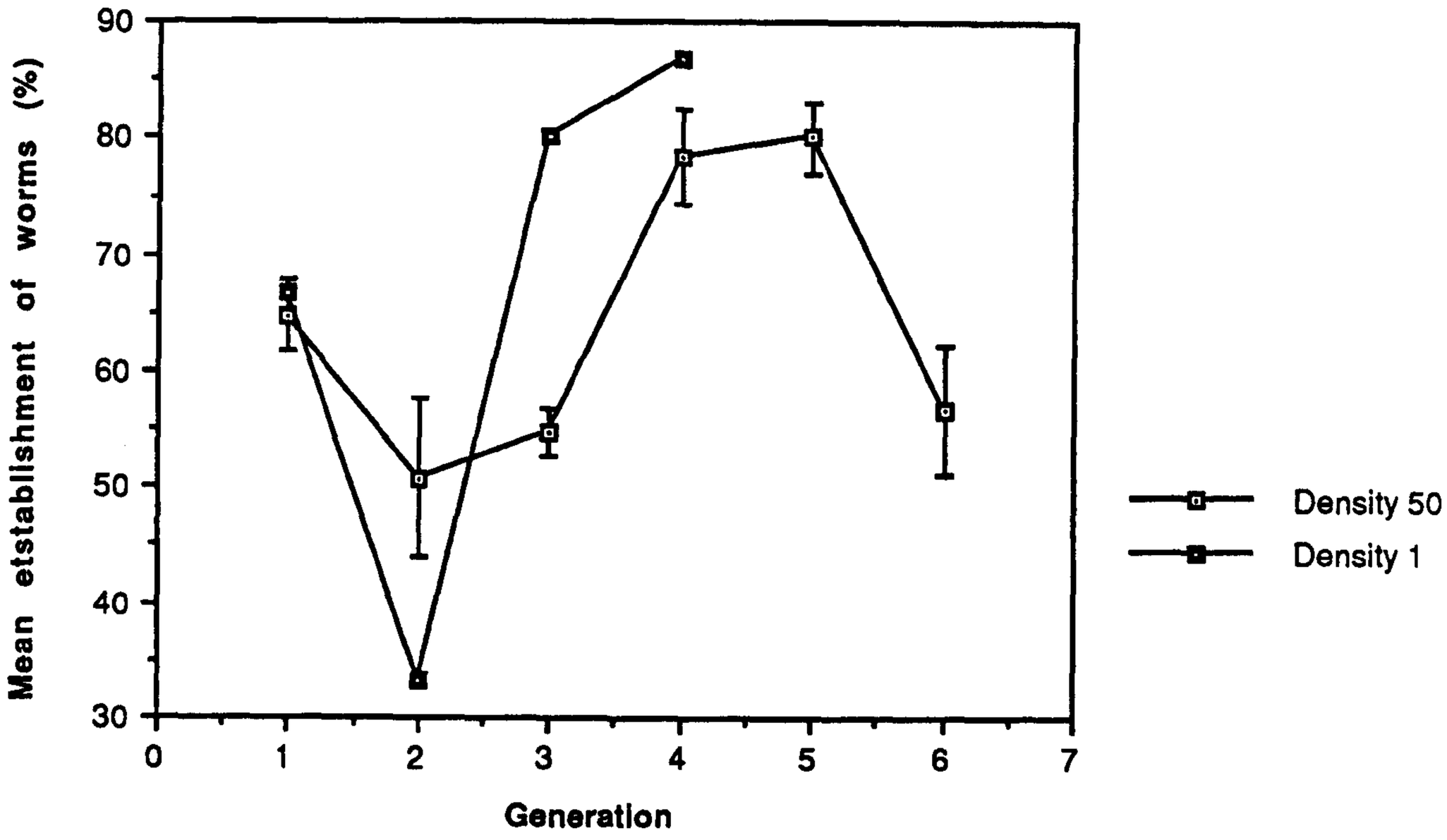

90
80
70
60
50
40
30
0
1
2
3
4
5
6
7
Mean etstablishment of worms (%)
Generation
Density 50
Density 1

Fig. 8.4

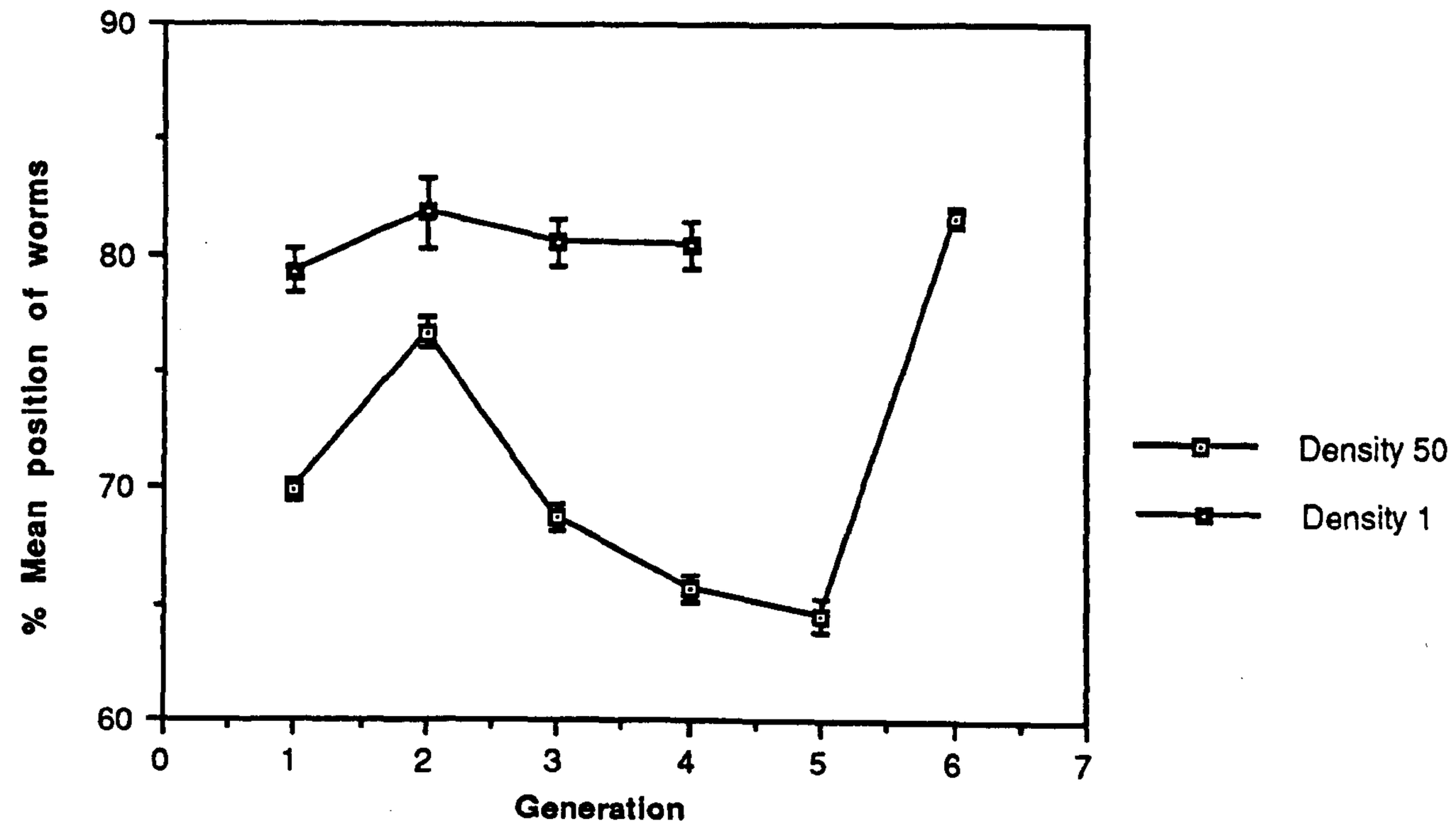

90
80
70
60
0
1
2
3
4
5
6
7
% Mean position of worms
Generation
Density 50
Density 1

Fig. 8.5 Mean length of worms at each successive generations (data points include standard errors)

Fig. 8.6 Mean width of worms at each successive generations (data points include standard errors)

Fig. 8.5

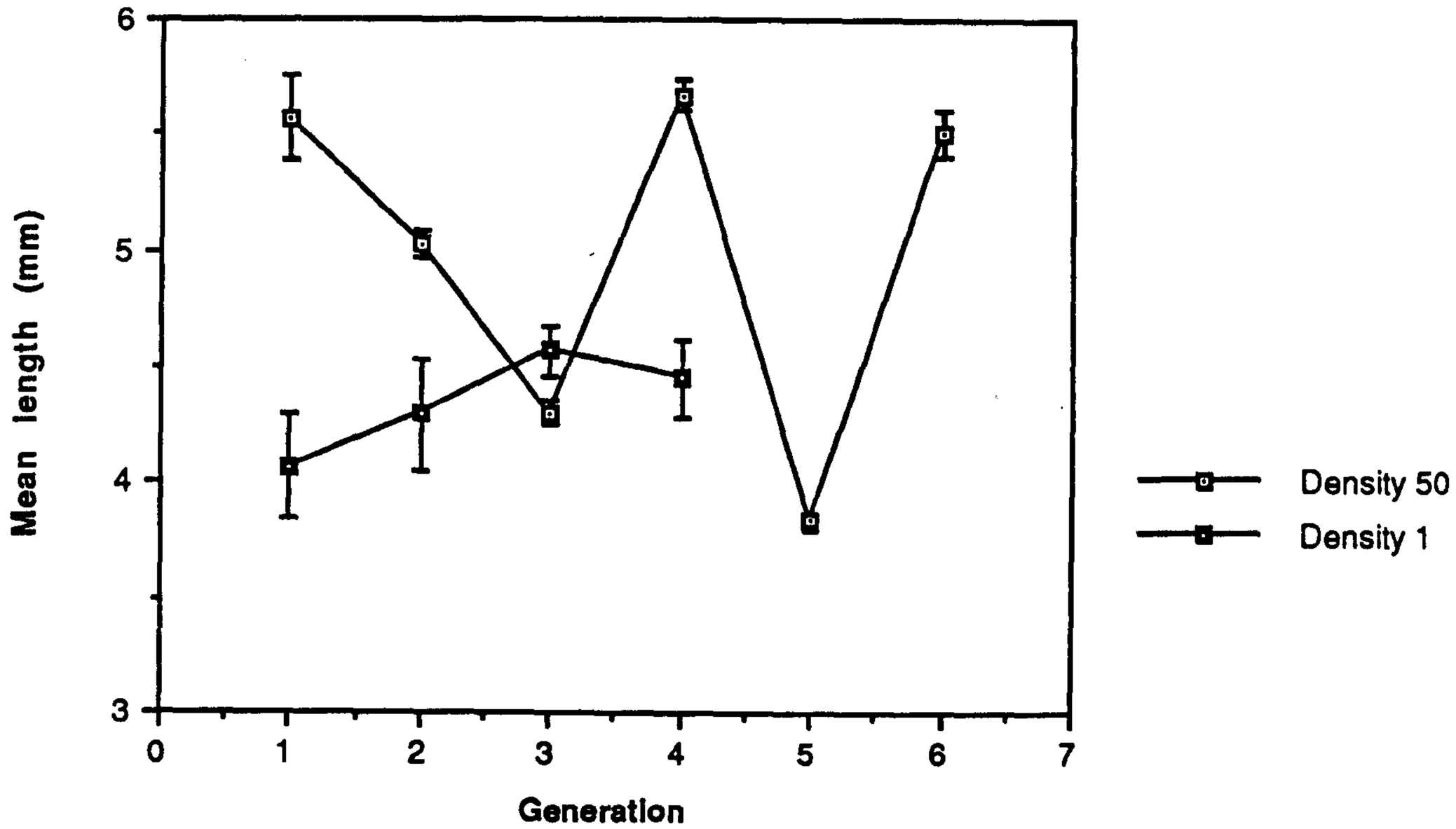
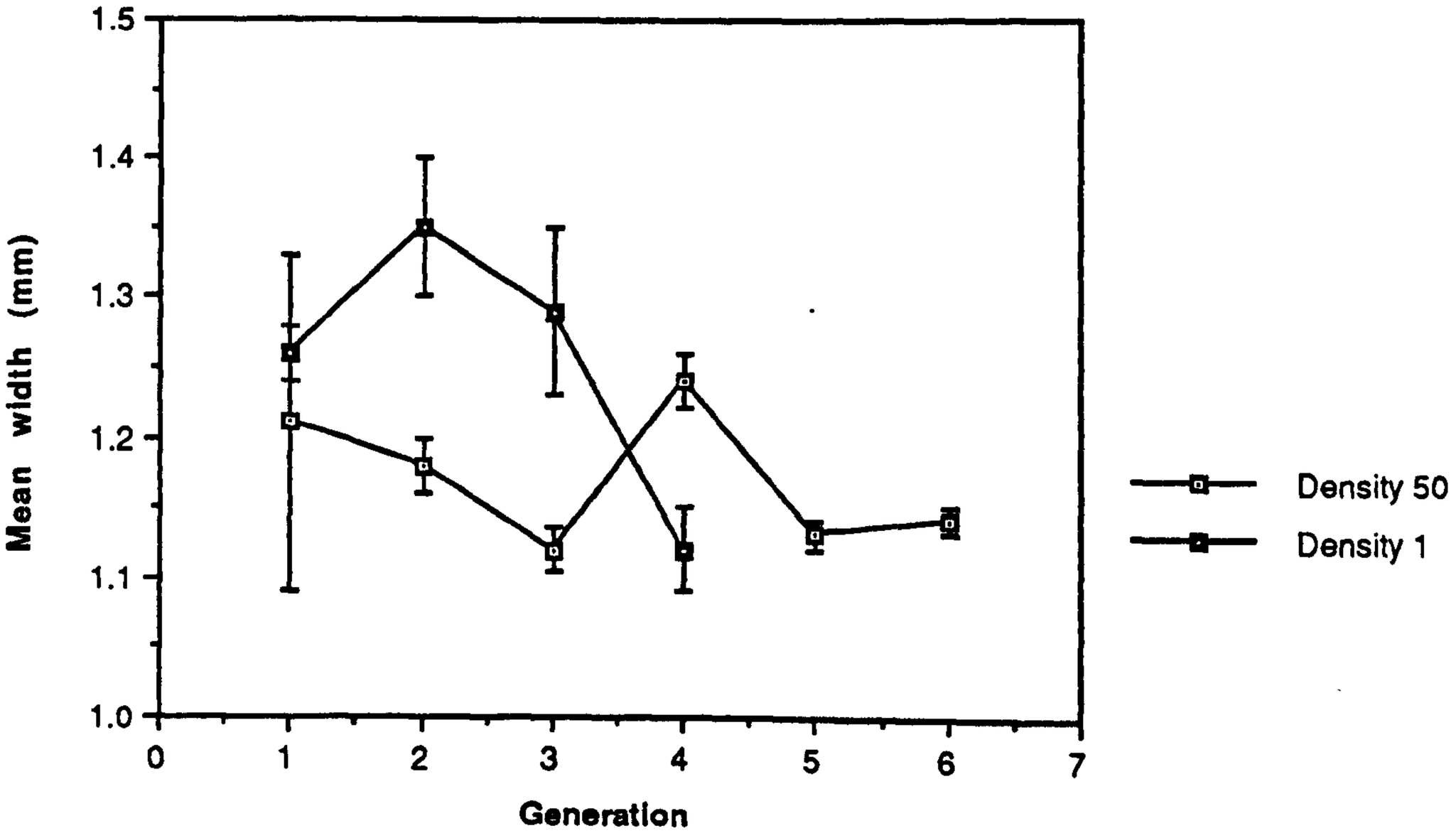

Mean length (mm)
6
5
4
3
0
1
2
3
4
5
6
7
Generation
Density 50
Density 1

Fig. 8.6


Mean width (mm)
1.5
1.4
1.3
1.2
1.1
1.0
0
1
2
3
4
5
6
7
Generation
Density 50
Density 1

8.3.4 Egg production per worm and intra-uterine eggs

From Fig. 8.7 it is apparent that egg production per worm in 24 hours is far greater in worms in the single line than those of the multiple line throughout the first four generations. In the multiple line egg production per day ranges from 147 to 219 eggs compared with a range from 433 to 638 in the single line worms. This overall result signifies a density-dependent constraint on egg output capability similar to that demonstrated in Chapter 6. Analysis revealed a very significant difference between the mean number of eggs expelled per worm in 24 hours by the multiple and single lines at each of the four successive generations (Generation 1, $t=5.42$, D.F.=10, $P<0.001$; Generation 2, $t=5.16$, D.F.=7, $P<0.01$; Generation 3, $t=6.02$, D.F.=8 $P<0.001$; Generation 4, $t=8.45$, D.F.=7, $P<0.001$). Despite this overall difference, by Generation 4 in the single line egg output had declined when compared to the previous three single line generation mean values.

With respect to the mean number of *in-utero* eggs no overall between- generation pattern was seen in the multiple line but in the single line *in-utero* egg values appeared to decline from Generation 2 onwards (Fig. 8.8). The mean number of eggs contained in the uteri of worms in the single line infection at Generation 1 (266) was significantly greater than the multiple line (97) at this same stage ($t=-4.90$, D.F.=14, $P<0.01$) and similarly at Generation 2 (single line, 297; multip[le line, 190; $t=-4.47$, D.F.=16 $P<0.001$) as revealed graphically in Fig. 8.8. In marked contrast at

Generation 3 these *in-utero* egg values had converged in both the single line (211) and the multiple line (216) revealing no significant difference between the two egg populations (t=0.20,D.F.=4,P>0.05). A similar pattern was observed at Generation 4 where the mean number of *in-utero* eggs in the multiple line was 125 compared to 161 in the single line (t=-1.89, D.F.=10, P>0.05).

To examine the relationship between the mean number of eggs per worm per 24 hours (EPW) and *in-utero* egg number (EPU) in the two infection lines Figs. 8.9(a) and (b) were plotted. In the multiple line infection there is a clear linear positive relationship between these two variables (N=59; R=0.45; P=<0.001) but no such relationship is present in the single line infection (N=17; R=0.35; P>0.05).

8.3.5 Percentage egg hatching success

The percentage success of egg hatching in the multiple line showed very little variability over the full six generation course of this experiment. Values of between 81% and 95% occurred in all generations (see Table 8.4). For the first three generations egg hatching success in the single line was very similar with values varying between 79% and 96% (see Table 8.4). At Generation 4, however, the single line demonstrated dramatic changes in this characteristic. Hatching success, which had been at 96% at the end of the third generation went down to 4% (see Fig 8.10). This collapse made it impossible to obtain sufficient miracidia to infect

Fig. 8.7 Mean egg production per worm per 24 hours at successive generations (data points include standard errors)

Fig. 8.8 Mean number of *in-utero* eggs per worm at successive generations (data points include standard errors)

Fig. 8.7

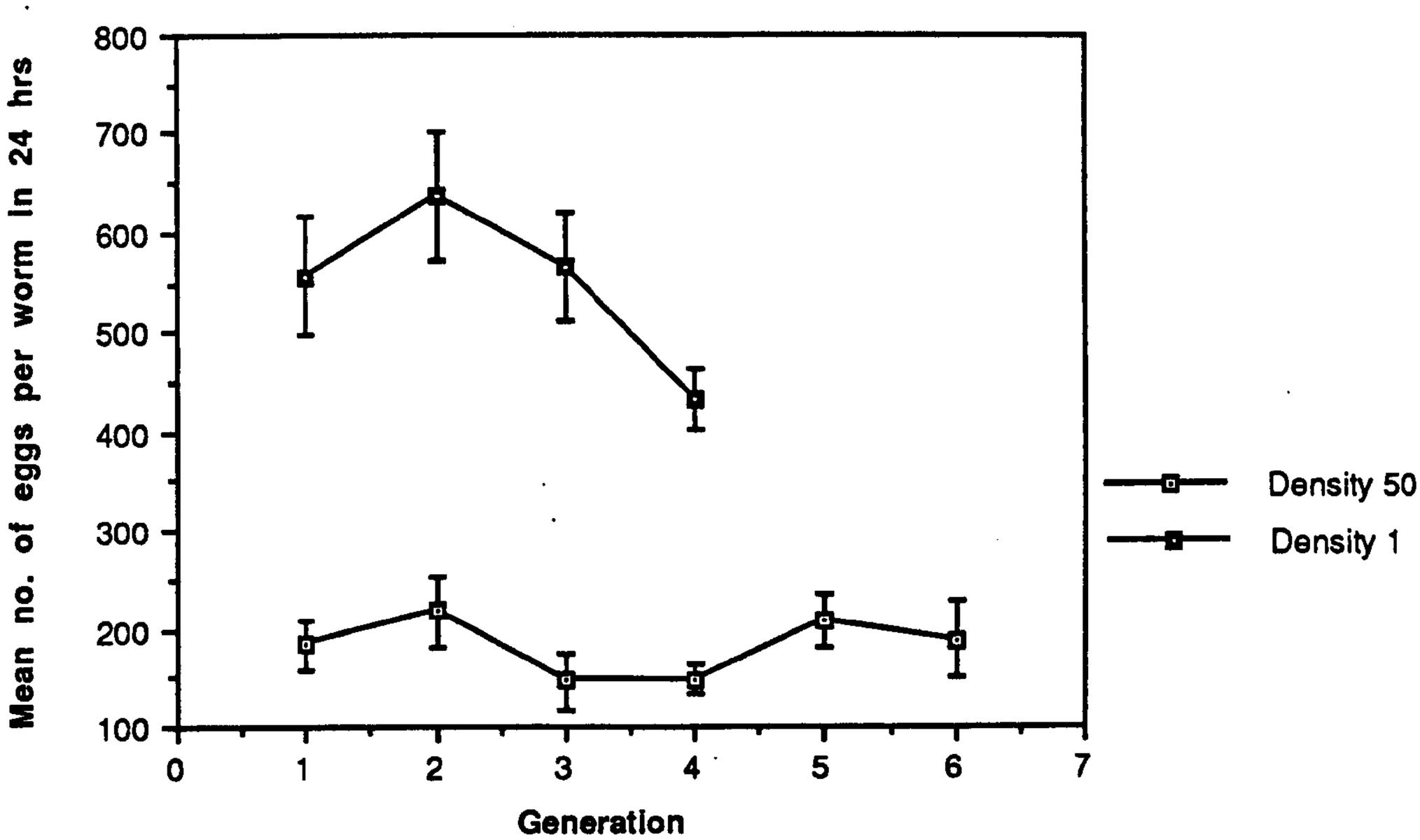

Mean no. of eggs per worm in 24 hrs
800
700
600
500
400
300
200
100
0
1
2
3
4
5
6
7
Generation
Density 50
Density 1

Fig. 8.8

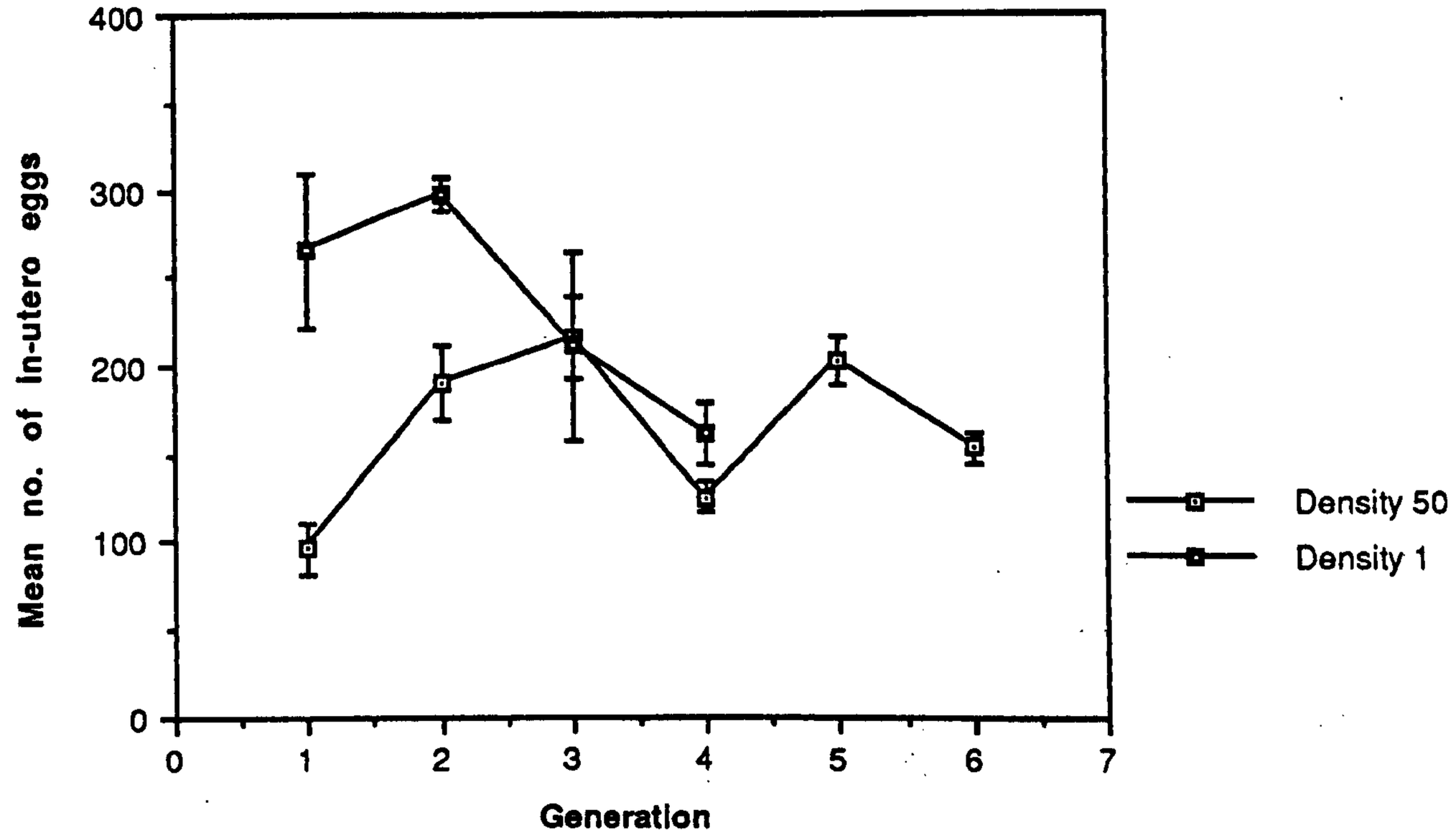

Mean no. of in-utero eggs
400
300
200
100
0
0
1
2
3
4
5
6
7
Generation
Density 50
Density 1

Fig. 8.9 (a) The relationship between EPW and EPU (Multiple line)

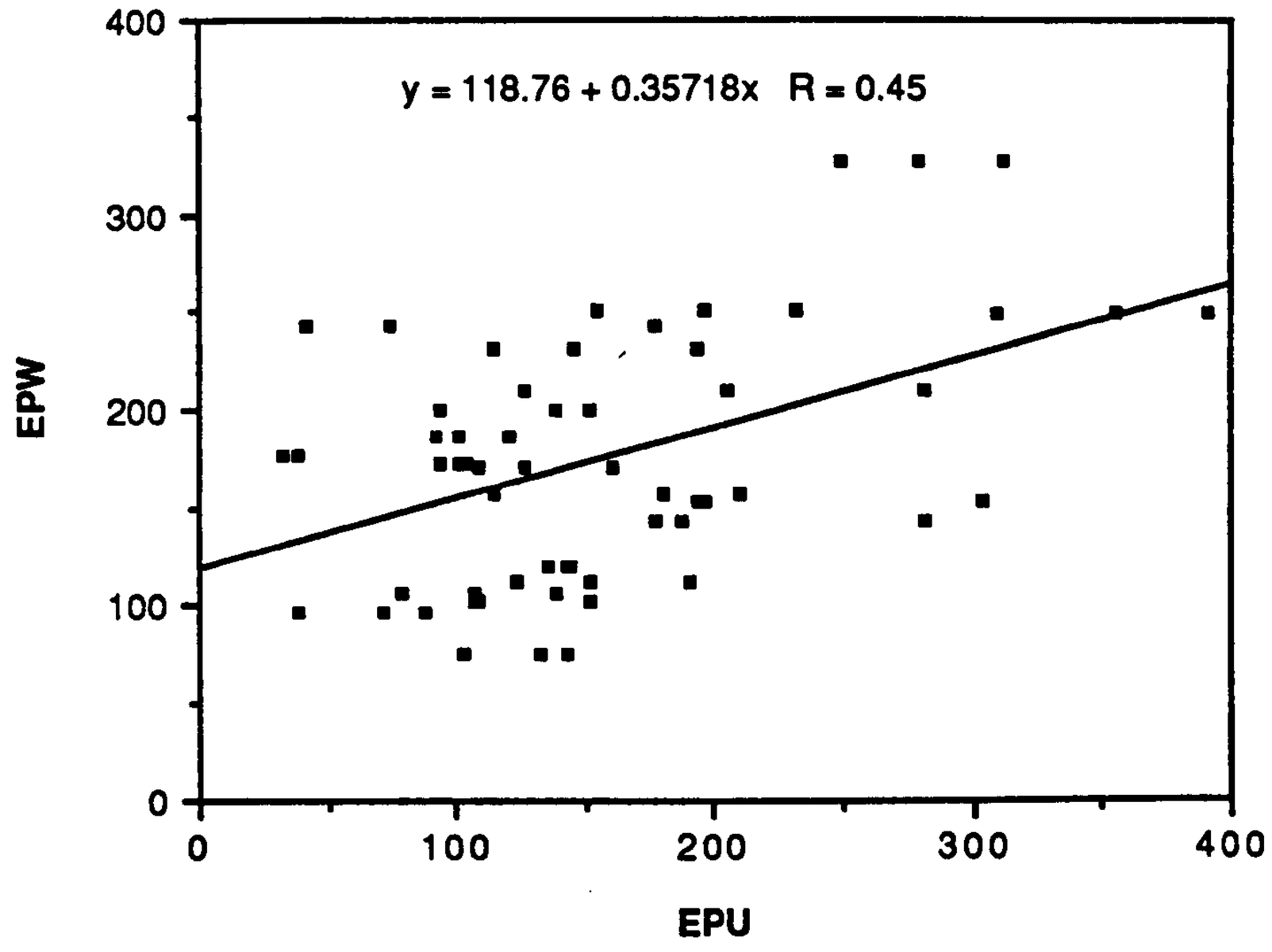


Fig. 8.9(b) The relationship between EPW and EPU (Single line)

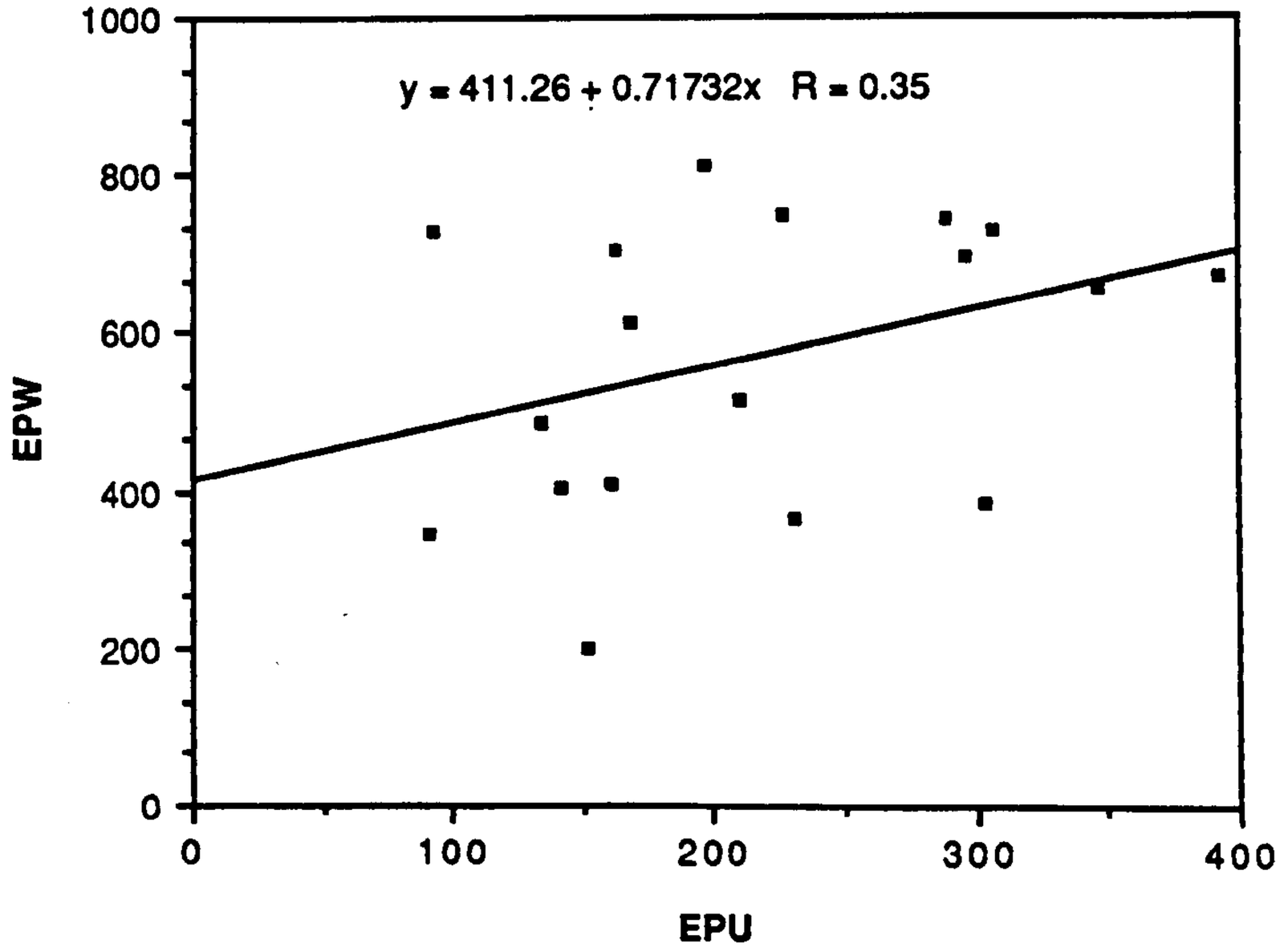


Fig. 8.10 Percentage egg hatching success at successive generations (data points include standard errors)

Fig. 8.11 Percentage miracidial infection success

Fig. 8.10

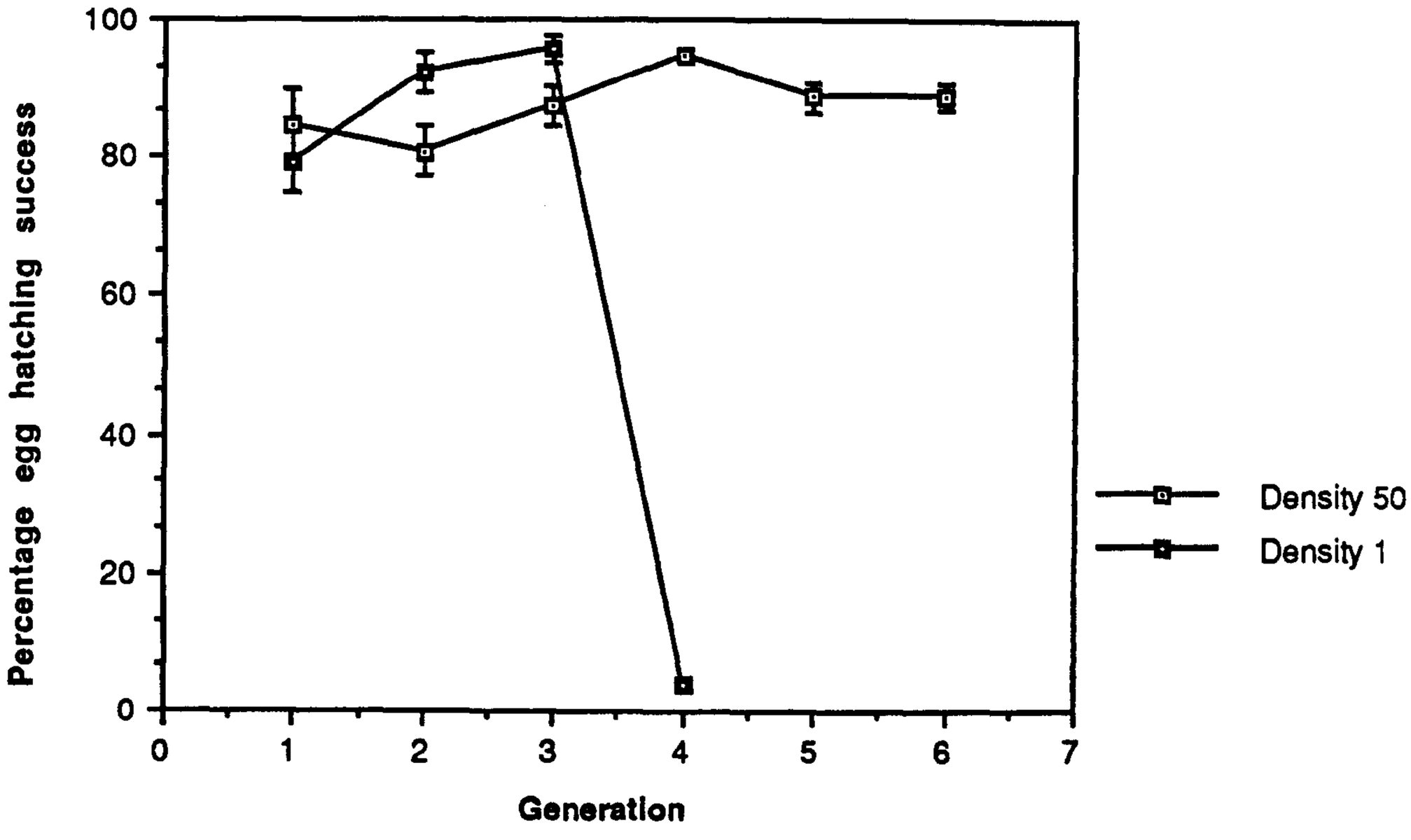
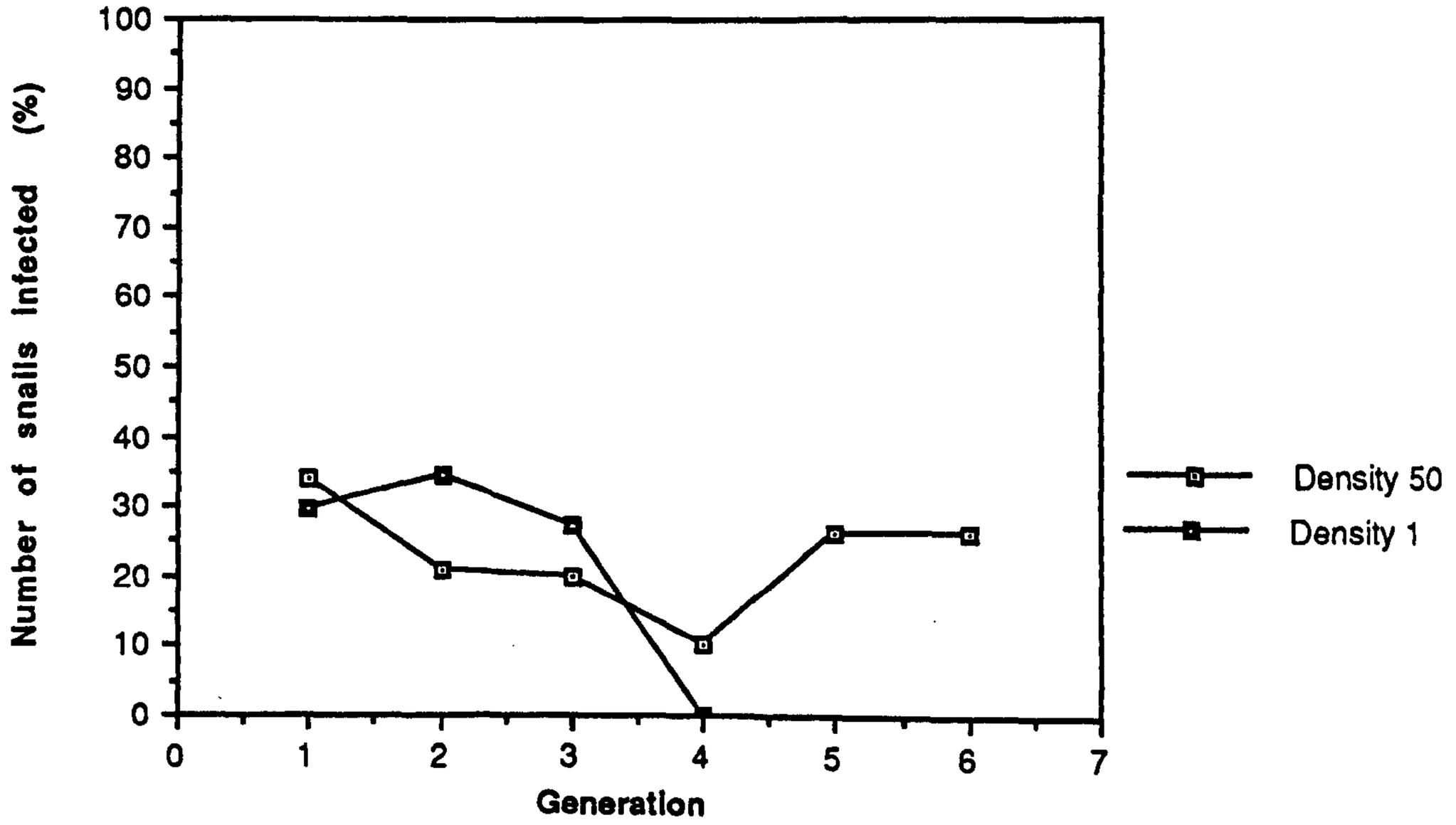


Fig. 8.11



snails. Of those snails that were exposed to those miracidia none became infected thus preventing the establishment of the next generation (see Fig 8.11). Eggs from this line failed to develop and in those eggs that did form fully mature miracidia, development was delayed by approximately 7 days. The eggs that did hatch exhibited an unpredictable hatching pattern.

8.3.6 Percentage miracidial infection success

The percentage of snails successfully infected by the standardized miracidial expossure technique varied over six generations between 10% and 34% in the multiple line with no trend apparent through these generations (see Table 8.4 and Fig. 8.11). Over the first three generations a similar pattern was observed in the single line with percentage infections of between 27% and 35% being noted (see Table 8.4 and Fig. 8.11). As explained in section 8.2.5 above, however, by Generation 4 the percentage infection success had dropped to zero.

8.3.7 Cross-fertilisation of single line worms from Generation 4

To investigate further the potential reproductive capacity of metacercarial cysts from Generation 4 of the single line, multiple infections with cysts of this type were carried out. Cysts were taken from a population derived from cercariae emitted from the same snail that produced all the cysts utilized to produce Generation 4 of the single line. Two mice (mouse A and mouse B)

were each infected with 50 metacercarial cysts of this type. The results of these infections are summarized in Table 8.5 and show interesting differences with both single and multiple line infections in Generation 4. The worms were smaller than those in either line at Generation 4 (see Tables 8.2 and 8.5). Their total egg production in 24 hours averaged about 1400 eggs, much more than seen in single Generation 4 worms but much lower than those in the multiple line. Per worm output (EPW), however, with a mean of about 41 eggs per worm per 24 hours was much lower than that associated with either line in Generation 4. A similar reduction was apparent in *in-utero* egg numbers (EPU), averaging only 70 eggs per uterus. These low EPW and EPU values are both associated with an extremely unusual retardation in worm sexual development in these two mice. In all previous infections by 10 days postinfection all worms were sexually mature, in mouse A and B about one third of these worms which established were immature. All eggs produced by worms in mouse A and B were non-viable.

8.3.8 Parallel single line infection with Generation 4 cysts

Using cysts of the same origin as those utilized in section 8.3.7, 10 mice were each infected with single cysts. This set of infections represented a parallel single line infection to that already described in the main experiment. The results of these infections are summarised in Table 8.5. In terms of most parameters this parallel single line infection behaved in a very similar fashion to the Generation 4 single line in the main experiment. Similar values for

mean worm positions, worm size, total eggs produced per 24 hours, EPW and EPU were noted (see Table 8.2 and 8.5). The eggs produced by these worms however behaved somewhat differently than those in the initial Generation 4 single line. The eggs developed slowly, delayed by about 7 days in all and in those egg which did hatch (68%), hatching was unpredictable with respect to the normal light stimulus. This hatching success however was much greater than that of the original Generataion 4 single line (4%). Despite this, the miracidia obtained in this parallel infection were completely unable to infect snails using the standard infection protocol.

8.4 Discussion

8.4.1 The effects of multi-generation self-insemination on *E. liei*

The initial success of the single line first generation indicates that *E. liei* in Swiss T.O. mice are capable of self-fertilisation under conditions which preclude cross-fertilisation. It is assumed, for the purpose of this Discussion section, that the only egg-generating process possible in single worm infections is self-fertilisation; that is, parthenogenesis is not occurring. This assumption will be considered in more detail and further justified in Chapter 9. With continual selfing it appears that *E. liei* is able to survive in a self-fertilising situation with few deleterious effects through at least three complete generations. This novel finding has not been

demonstrated previously in any echinostome system although self-fertilisation in single worm infections of echinostomes and the ability of miracidia derived from such infections to infect snails have both been demonstrated before (Fried *et al.*, 1988). The following sections discuss the different aspects of reproductive success investigated in this Chapter in the two infection lines.

Establishment success

In this study, throughout the first four generations, establishment success was highly variable in both single and multiple worm infections. The generation to generation variation in both lines gives the impression of being random deviations from a similar mean value in each line at around 68% establishment success. It is of interest that the single line value in Generation 4, the generation which demonstrated a dramatic fall in egg production has the highest establishment success of all the four generations at 87%. Evidently the deleterious changes that occurred in Generation 4 in the single line did not extend to establishment efficiency of the juvenile worms from metacercarial cysts.

Worm positions

Throughout the first four successive generations, the single worm infections tended to inhabit a more posterior position in the small intestine when compared with multiple worm infections. This difference in mean attachment position must be compared with the earlier findings in Chapter 6 (see section 6.4.2) on worm density

and mean gut position. These findings show that with increasing exposure density (6-100 cysts), the resulting worm populations utilized the same general percentage regions of the gut when observed at the same times postinfection. The differences observed in this Chapter would not have been apparent in the study carried out for Chapter 6 because there a less precise analysis of gut positions was carried out. The significantly more posterior locations of the single worms found in the present experiment could be due to a number of influences;

1. a real density-dependent effect
2. a genetic difference between the two lines which showed itself as different attachment preferences
3. a consequence of the findings by Franco, Huffman and Fried (1988) that increased dosage levels of metacercarial cysts enlarged the overall spatial distribution of the parasites in the gut. This could only produce the observed effect if the spread was unilateral.

Worm dimensions

The findings of Chapter 6 (see section 6.4.3) demonstrated that increasing adult worm densities in the mouse gut had no consistent effect on worm body dimensions. Worm size was not, apparently, affected by density-dependent constraints. The findings on worm size in this Chapter appear to confirm this finding (see Tables 8.1 and 8.2; Figs. 8.5 and 8.6). Equally there is no evidence that genetic differences between the two lines has induced size differences between them.

Egg output per worm in 24 hours (EPW)

The number of eggs expelled per worm in 24 hours was not a subject of any of Fried's single worm infection studies (Fried and Alenick, 1981; Fried, Huffman and Franco, 1988; Fried and Sousa, 1990) and so there is no other echinostome data of this sort to compare with that of this study. Here, the overall 24 hour egg output by each worm population was far greater in the multiple worm infections than the single worm infections but the single worm infections expelled the greater number of eggs per worm in 24 hours at each successive generation. These results are a conclusive indication of the marked density-dependent constraint on this variable throughout successive generations. From Table 8.6 it can be seen from the ratio of EPW for the single line to EPW for the multiple line that the single worms expell a mean of 3.18 times the number of eggs produced by the multiple line worms. This implies that mean egg output per worm over 24 hours is greater in the single line throughout the duration of the single line infections.

In-utero **egg numbers and egg output capability**

The number of *in-utero* eggs was also significantly greater in single worms than in multiple line worms at both Generations 1 and 2. Interestingly there was no significant difference in Generation 3 and 4. The former result (Generation 1 and 2) would conform with the density-dependent effects related to worm burden and mean uterine egg number (where the uterine carrying

capacity of worms in higher density infections is reduced) determined in Chapter 6 (see section 6.4.4). The latter, (Generation 3 and 4) including the apparently significant drop in the mean egg production per worm in 24 hours at Generation 4 in the single line, (see Fig. 8.7), is a probable indication of a genetic deficit caused by continued self-insemination.

Table 8.6 shows that single worms are able to expell a mean value of 2.4 times the number of eggs present in the uterus per day compared to 1.2 in the multiple line at each generation. This indicates a facet of the egg output capability in the multiple line infection which shows a density dependent constraint, a feature similar to those observed in increased density infections in Chapter 6. Interestingly, there is a strong correlation between mean egg production per worm per 24 hours and *in-utero* egg number in the multiple line only (Figs. 8.9(a) and (b)). In Chapter 6 this relationship was considered over a range of densities and the results appeared to justify the assumption that the number of stored eggs in a uterus could be used as a good indirect measure of egg production rate. The multiple line infection in the present experiment appears to conform to this relationship but the single line does not. The lack of correlation in the single line may imply that the relationship only operates at specific initial metacercarial cyst exposure densities or that successive generations of self-insemination can play a part in eliminating the relationship.

TABLE 8.6 The egg output capabilities of worms at successive generations

Generation	Multiple line EPW/EPU*	Single line EPW/EPU	EPWsingle /EPWmultiple
1	1.90	2.09	3.02
2	1.18	2.15	2.91
3	0.68	2.68	3.86
4	1.18	2.69	2.92
5	1.04		
6	1.24		
Mean values	**1.20**	**2.40**	**3.18**

* EPW =mean egg production per worm per 24 hours
EPU= mean in-utero egg number

Alternatively the lack of correlation could be due to the smaller sample size (18 v. 60) in the single line analysis.

Egg hatching and miracidial infectivity

The collapse in the percentage success of egg hatching in the single line at Generation 4 was mirrored by the virtually similar result in the parallel single line infection. In both cases miracidial infectivity was zero.

Reproductive capability of multiple infection single line cysts

The multiple infection involving the single line cysts of Generation 4 produced both immature worms and eggs that completely failed to develop (see Table 8.5). These worms appeared to be unable to match the reproductive capability of the worms from the outbred multiple line infections. The EPW and EPU values of the multiple infections with single infection line-derived worms were even lower than those from Generation 4 of the single line infection. The high proportion of immature worms recovered in these infections and their very much depressed reproductive capability was very unusual and could not be accounted for by density-dependent mechanisms. These deficits pointed instead to an underlying deficient genetic character associated with the worms from the single line of the fourth generation. Even when delivered as a multiple infection, development of these worms generated no gene flow at all into the next generation. It is interesting that cross-

insemination opportunities provided after four generations of selfing, not only cannot relieve the reproductive deficits of the single infection line, they appear to increase them.

8.4.2 Monometacercarial cyst infections within the Echinostomatidae

It is very difficult to produce an overall summary of studies on monometacercarial cyst infections with echinostomatids which is usefully comparative because of the marked variations in experimental design and the varying densities used within these experiments to establish comparative multiple infections. Fried *et al.* (1988) demonstrated that single worms of *E. revolutum* grown in hamsters were able to produce miracidia that infected laboratory reared *Helisoma trivolvis* snails to produce patent infections. This ability of *E. revolutum* to self-fertilise was also demonstrated by Fried and Alenick (1981) who infected chicks with single metacercarial cysts of *E. revolutum,* although in this work the ability of miracidia to infects snails was not examined. Fried and Alenick (1981) noted that in the chicks fed single cysts of *E. revolutum*, establishment success was much retarded when compared to multiple infections, finding that out of the 53 chicks administered single cysts only 3 (5.7%) became infected. They observed that worms from multiple infections (chicks administered 2, 5 and 10 cysts) tended to locate more posteriorly in the intestine than single worms as single worms were found only in the ileum whereas multiple worms were recovered in both the ileum and

rectum. They also noted that length measurements of multiple and single worms were similar. Along with this they also observed that the number of *in-utero* eggs and the percentage egg hatching success were considerably greater in multiple than in single worm infections. Fried *et al.* (1988) stated that establishment success of single worm infections of *E. revolutum* was greater in hamsters (50%) when compared with the chick (5.7%). Fried *et al.* (1988) found that in multiple infections (hamsters administered 5 cysts) of *E. revolutum*, worms extended their distribution both anteriorly and posteriorly. They found no difference in mean length of single versus multiple worms but did observe that mean width of single worms was significantly greater than that of multiple worms. They also observed that *in-utero* egg counts, the percentage egg hatching success and the infectivity of miracidia in snails were similar in both single and multiple infections. A further study carried out involving monometacercarial cyst infections of *E. caproni* in ICR mice (Fried and Sousa, 1990) showed that worms from multiple infections (5 metacercarial cysts) at 14 days postexposure contained twice the number of eggs per uterus than single worms at the same time period. Body area measurements of 21 and 28 day old worms from multiple infections were also found to be significantly greater than that of single worms of the same age. Conversely, a study comparing monometacercarial cyst infections and multiple worm infections (2 and 5 metacercarial cyst) of *E. caproni* in golden hamsters showed that the mean number of *in-utero* eggs from single worm infections was

significantly greater than worms from multiple infecions at the same time period (Fried, Huffman and Weiss, 1990). This study also demonstrated that miracidia derived from single worm infections of *E. caproni* were capable of infecting laboratory reared *B. glabrata* and producing patent rediae.

It is obvious that there has been great variability in the apparent impact of echinostome selfing on measures of reproductive success in different parasite species, tested in different hosts. None of these past studies however have continued with a selfing programme further than the infection of snails in Generation 2. It is therefore very difficult to extrapolate their findings into the area of investigation considered in this Chapter.

8.4.3 Self-fertilisation as a mode of reproduction in digeneans

Most work carried out on monometacercarial cyst infections with echinostomes has involved a single generation of such infection as described by Fried and Alenick (1981), Fried *et al.* (1988) and Fried and Sousa (1990). Table 8.7 summarises comparative monometacercarial and multiple cyst infections carried out in non-echinostome digeneans. Nollen (1971a) noted that there was no difference in the rate of maturation in monometacercarial infections of *Philophthalmus megalurus* in chicks when compared with 5 worm infections. Over 60 days the growth in length of worms from both single and multiple infections was comparable

TABLE 8.7 Summary of monometacercarial cyst infections in non-echinostome digeneans (see Discussion section 8.4)

Parasite	Zygocotyle lunata	Zygocotyle lunata	Leucochloridiomorpha constantiae	Paragonimus kellicotti	Paragonimus ohirai
Host	chick	chick	chicks	cats	cats
Cyst density					
Single	1	1	1	1	1
Multiple	10		2		
Size difference	no significant difference		not tested		
Ability of eggs from single infections to hatch	yes	yes	not tested	no	yes
*Ability of miracidia to infect snail host	not tested	yes	not tested		no
*Ability of cysts to infect definitive host at Generation 2	not tested	yes	not tested		
Author	Nelson and Fried (1970)	Bacha (1966)	Fried and Harris (1971)	Sogandares-Bernal (1966)	Hashiguchi Takei and Miyazaki (1969)

*From single infections

TABLE 8.7 (cont.) Summary of monometacercarial cyst infections in non-echinostome digeneans

Parasite	Philophthalmus megalurus	Philophthalmus hegeneri	Philophthalmus hegeneri	Paragonimus westermani (Taiwanese)	Paragonimus pulmonalis	Paragonimus westermani (Japanese)
Host	chick	chick	chicks	kittens and puppies	cats	cats
Cyst density						
Single	1	1	1	1		1
Multiple	5		5			
Size difference	no significant difference		singles smaller			
Ability of eggs from single infections to hatch	yes	no	no	yes	yes	no
*Ability of miracidia to infect snail host	yes			not tested	not tested	
*Ability of cysts to infect definitive host at Generation 2	yes			not tested	not tested	
Author	Nollen (1971a) Nollen (1971b)	Fried (1962)	Colgan and Nollen (1977)	Fan and Chiang (1970)	Miyazaki Terasaki Habe (1981)	Miyazaki Terasaki Habe (1981)

*From single infections

and there was evidence for egg and sperm production as well as insemination in the single metacercarial cyst infections. Nollen (1971a) tested the viability of the miracidia by exposing them to *Pleurocera acuta* and *Goniabasis* sp. snails. Five out of 14 snails became infected and shed cercariae forming metacercarial cysts before dying. Nollen (1971a) concluded that *P. megalurus* could survive successfully through at least one passage as a self-fertilising strain. A later study by Nollen (1971b) showed that a self-fertilising strain of *P. megalurus* could be carried through 3 successive life cycles with little or no deleterious effects when compared with cross-fertilising strains. The experimental design adopted by Nollen was different to that described in this study as larval forms at both miracidial and cercarial infection stages, from single infections were allowed to mix. No significant differences were noted in the parameters of growth, eggshell formation and the viability of miracidia and cercariae. Significant reductions in recovery rates of adult worms from monometacercarial cyst infections were found in the 2nd and 3rd generations. Total recovery in the 2nd generation being 19% and 46% from the single and multiple line respectivly. In the third generation the recovery rates for the single line were 22% compared with 45% for the multiple line. The experiment ended at the end of the third generation when snails (*P. acuta*) exposed to miracidia died. During this study, it was also noted that recovery rates of adults in multiple infections derived from a line that had self-fertilised for two generations were similar to those of multiple infections derived

from a cross-fertilising line. Nollen, summarising these results in the context of the ecology of this digenean, believed that the ability of *P. megalurus* to survive in a self-fertilising mode was an adaptation by this parasite for survival in an environment where hosts were scarce or the infective stages were scattered by a fast flowing stream.

Nelson and Fried (1979) observed growth and reproduction in single and 10 worm infections of the digenean *Zygocotyle lunata* in chicks. They found that the average percentages of worms recovered from single and multiple worm infections were 36% and 29% respectively and noted that worms recovered from single and multiple 7, 14, 21 and 28 day infections did not differ significantly in length. They noted no significant differences in the number of eggs produced in the two infection lines (that is, eggs produced per worm in one hour, *in vitro*) and commented on the fact that eggs from single worm infections contained fewer fully developed miracidia and hatched less successfully than eggs resulting from worms from multiple infections. Histological observations in this study revealed that the seminal vessicles of these single worms were filled with spermatozoa.

Bacha (1966) carried out an earlier study involving monometacercarial cyst infections of *Z. lunata* in rats. These single worm infections produced eggs which released miracidia and were used to infect 60 laboratory raised snails (*Helisoma* sp.). Only 1

snail became infected and cysts from this snail were administered to 7 rats, 6 receiving 1 metacercariae the remaining rat 25. Worms recovered at 40 days postinfection were all found to contain eggs apart from one single worm infection. The miracidia from single worm infections died prior to hatching but eggs obtained from the rat with 25 cysts released miracidia which failed to infect snails. Bacha proposed that self-fertilisation or possibly parthenogenesis had resulted in genetic disturbances responsible for the apparent loss of miracidial viability.

Fan and Chiang (1970) demonstrated clearly that a single worm of the Taiwanese lung fluke *Paragonimus westermani* could develop to sexual maturity in the lungs of kittens and puppies. They demonstrated that the eggs were viable, releasing miracidia with the hatching rate of eggs from puppies being 9% and 12% to 23% from kittens. These workers did not test the viability of released miracidia through to the adult stage of development. They used the finding of eggs *in-utero*, in the lung cyst, as well as the surrounding tissue, as a criterion for sexual maturity and found that 65% of the worms recovered were mature, and the remaining 35% immature. Fan and Chiang speculated on the possibility of biological differences between separated geographical populations of *P. westermani* because earlier workers had concluded, after observing a lack of development in *P. westermani* in single worm infections, that complete maturation of this parasite could only be brought about by cross-fertilisation, that is, that previous single

worm infections had produced immature worms (Yokogawa, Yoshimura and Oshima, 1960; Yokogawa, Cort and Kokogawa, 1960).

Miyazaki, Terasaki and Habe (1981) compared single worm infections of the Japanese strains of *Paragonimus westermani* and *Paragonimus pulmonalis* in cats. They observed that *P. pulmonalis* fully matured in cats and had numerous eggs in the uterus, but noted no sperms in the male reproductive organs and in the seminal receptacle except for a small number of abnormal sperms in the former. This species produced eggs which showed a high percentage development success and which released active miracidia. With respect to single infections of *P. westermani* which produced fully mature worms which were smaller, worms contained numerous sperms in the male reproductive organs but lacked sperms in the seminal receptacle. *P. westermani* laid unfertilised eggs which hardly developed and those that did develop were impossible to hatch. All worms of *P. westermani* were found crawling in the pleural cavity in contrast to *P. pulmonalis* which was located in the lungs. These workers explained that this behaviour was due to the biological character of the bisexual type, that is, that *P. westermani* have to exchange their sperms by pairing in the worm cyst and therefore they keep migrating until they meet their mate. They concluded that a single adult of *P. pulmonalis* can make the worm cyst without a partner and produce viable eggs without sperm, while *P. westermani* does not make a worm cyst until it meets a partner because cross-

fertilisation is indispensible for *P. westermani* in order to lay viable eggs.

Fried and Harris (1971) demonstrated that monometacercarial cyst infections of the digenean *Leucochloridiomorpha constantiae* in the domestic chick produced adults capable of self-insemination and they compared the viability of these single infections with double infections. They observed that eggs were present and fully developed in all worms from double infections from 7 to 10 days but only observed developed eggs in single worms at 9 and 10 days postinfection. The overall mean number of *in-utero* eggs per worm in double infections was found to be approximately twice that in single worm infections. The viabilty of the hatched eggs and miracidial infectivity were not studied. In this study Fried and Harris observed autocopulation, (that is, the insertion of the cirrus into the metraterm) in single worm infections while in the double worm infections they noted that some worms were in cross-copulation but did not observe autocopulation.

8.4.4 Reproductive behaviour in helminths utilizing self and cross-fertilisation

In the Monogenea, both cross and self-fertilisation are known to occur (Smyth and Halton, 1983). Tinsley and Owen (1975) have shown that the monogenean *Protopolystoma xenopodis* introduced at a density of one into the urinary bladders of laboratory reared *Xenopus*, are able to self-fertilise.

Self-insemination has also been demonstrated *in-vitro* in a number of cestodes such as *Schistocephalus solidus* (Smyth, 1954) and *Spirometra* sp. (Berntzen and Mueller, 1972). While Smyth and Smyth (1969) found exclusive self-insemination in *Echinococus granulosus* from dogs. Cross-insemination in cestodes has been described by Cox, Ciordia and Jones (1956) in *Hymenolepis serrula* and by Williams and McVicar (1968) in *Acanthobothrium quadripartitum* and *Phyllobothrium* sp. Nollen (1975) transplanted individual worms of *Hymenolepis diminuta* labelled with ^{3}H-thymidine either as single infections in rats or into multiple infections and demonstrated that in the single infections self-insemination occurred while in the multiple infections both self-insemination as well as cross-insemination was occurring. Labelled sperms were found in the seminal receptacle of as many as 60 consecutive proglottids in both infections. Hyman (1951) described self-fertilisation in cestodes as taking place by the eversion of the cirrus into the vagina of the same proglottid. It appears from the experimental studies that self and cross-fertilisation are common in various species of cestodes. The effects of continual self-fertilisation in cestodes has been studied by Roger and Ulmer (1962) who maintained *Hymenolepis nana* in a self-fertilising strain for five generations. They noted an increased frequency of cystercercoid abnormalities, fewer eggs developed into cystercercoids in the intermediate host and fewer cystercercoids established themselves as adults in mice. No differences in the size of adults or

cystercercoids were found between selfing and crossing strains. Interestingly, Jones, Fitzgerald, Proffitt, Tan and Ward (1971) were able to maintain a selfing strain of *Hymenolepis microstoma* for 14 generations with no loss of viability.

It is now assumed that cross-fertilisation in digeneans is the common form of sexual reproduction but that self-fertilisation can also occur (Smyth and Halton, 1983). In general, copulation in digeneans occurs by the insertion of the cirrus into the metraterm of a neighbouring partner (Smyth and Halton, 1983). From the information already described it appears that self-fertilisation is an ability confined to specific species of digeneans.

Nollen (1968) placed *Philophthalmus megalurus* containing sperms labelled with ^{3}H-thymidine in the eyes of non-infected birds singly and with non-labelled worms to examine the frequency of self and cross-fertilisation. When 37 labelled worms implanted singly were recovered, 28 were found to have inseminated themslves as demonstrated by radioactive sperms in the female system. In multiple infections, the 33 labelled adults inseminated 47 out of 61 non-labelled adults. Mosely and Nollen (1973) transplanted ^{3}H-tyrosine labelled worms of *Philophthalmus hegeneri* from mutiple infections singly into the eyes of uninfected chicks and to observe cross-insemination 1 labelled worm was placed with 1 to 3 unlabelled worms per eye. Those transplanted alone did not self-fertilise but cross-fertilisation occurred in those un-labelled worms

exposed to labelled worms. Mosely and Nollen concluded that both *P. hegeneri* and *P. megalurus* will exclusively cross-inseminate in multiple infections. Further work by Nollen and Pyne (1979) involved the exposure of the frog digenean *Megalodiscus temperatus* to ^{3}H-adenosine. They observed that this worm in the frog's intestine was able to both cross and self-inseminate in multiple infections. Nollen and Pyne explained that the inseminative behaviour of the digenean *M. temperatus* was much like that of the cestode *H. diminuta* in that both self and cross-insemination occur in a group of worms within the same host. Mosely and Nollen (1973) explained that species of *Philophthalmus*, cross-inseminated only when in a group and never self-inseminated in a group situation. As self-fertilisation does not occur at all in some digeneans, it would seem that each species has acquired reproductive behavioural patterns peculiar to itself. The evidence using autoradiographic methods with *P. hegeneri* that suggests it does not self-inseminate supports Fried's (1962) observation that adults of this species grown in monometacercarial infections contain no sperm in their seminal receptacles and never produce viable eggs. Fried also showed that worms grown in isolation after 20 days stopped growing. In other species it has also been observed that cross-insemination is necessary for the development of ovigerous worms.

Sogandares-Bernal (1966) for instance, observed that in monometacercarial cyst infections with *Paragonimus kellicotti* in

the cat, the worms never developed past the wandering pre-adult stage. Similarly Hashiguchi, Takei and Miyazaki (1969) found that monometacercarial cyst infections of *Paragonimus ohirai* would develop in the lungs of rats to egg-laying adults but without cyst formation. They found that the miracidia within these eggs were non-viable. Colgan and Nollen (1977) demonstrated that *P. hegeneri* is incapable of self-fertilisation and single worms were found to be significantly smaller than worms from multiple infections. It is evident that in some single worm infections of various digeneans normal growth and development does not occur. A similar phenomenon has been noted in unisexual female infections of the dioecious schistosomes including *Schistosomatium douthitti* (Short, 1952), *Schistosoma japonicum*, *Schistosoma haematobium* (Armstrong, 1965) and *S. mansoni* (Michaels, 1969) where the presence of a male is required for normal growth and sexual maturity.

It would seem from the present study that, at least at intervals, multiple infections in which cross-fertilisation can take place are required by *E. liei* in order to produce fertile and viable eggs. It appears that *E. liei* does have the ability to use selfing for a specified time period with no apparent deleterious effects although the viability of the miracidia is diminished drastically after four generations of selfing. Other measures of reproductive success like the mean number of uterine eggs seemed to display deleterious changes before Generation 4. In summary, it must be concluded

that stringently maintained selfing in single line infections induced genetic deficits at some points between Generation 1 and Generation 4. With the available metacercarial cysts that produced the unsuccessful single worms of the fourth generation a number of additional experimental procedures were undertaken to determine the nature of these deficits and their phenotypic effects. These analyses are documented in Chapter 9.

CHAPTER 9

STRUCTURAL AND GENETIC CORRELATES OF LOW REPRODUCTIVE SUCCESS IN A *ECHINOSTOMA LIEI* SELF-FERTILISING LINE

9.1 Introduction

In Chapter 8, continual selfing of the offspring of a self-inseminating line of *E. liei* gave rise to a population of worms that produced eggs in which the hatching success was markedly depressed and a very large percentage of eggs failed to develop. Miracidia released from the small number of eggs that did develop failed to infect *B. glabrata* snails. This particular self-fertilising line was produced after four successive generations of inbreeding. Owing to the small number of available metacercarial cysts of this inbred line and the ever-present threat of the death and loss of snails harbouring these metacercarial cysts, a number of methods were utilized at the microscopical and biochemical level to test for any differences between the two lines with special reference to the low developmental and infective success of the eggs from the fourth generation single line.

It is possible that the cumulative effects of continual selfing could be responsible for the markedly depressed values for miracidial hatching and infection success exhibited by the single line worms at the end of the fourth generation. This can be assumed because of the continued reproductive success of the multiple line infection over six generations. It can be speculated that in multiple infections, assuming that cross-insemination is the norm, certain genetic advantages must operate which ensure the successful continuation of the reproductive line. With respect to miracidial hatching and infectivity success these capabilities appear to be lost after 4 generations of the single line. Therefore, to determine why

there is a loss of viability of *E. liei* after 4 successive generations in the single line, the following experimental techniques were carried out. Sections of these fourth generation worms and worms from the multiple line were examined at the light and electron microscopic level, chromosome squashes were employed to determine any chromosomal abnormalities and isoelectric focusing techniques were carried out to analyse some of the isoenzyme patterns of worms from both lines. Isoelectric focusing was carried out in an attempt to determine if there was any distinct genetic differentiation between worms from the multiple line and worms produced at the fourth generation of the single line.

9.2 Materials and Methods

9.2.1 Re-establishment of the single line fourth generation infection

To produce worms of the fourth generation, cysts were taken from a population derived from cercariae emitted from the same snail that produced all the cysts utilized to produce Generation 4 (see section 8.2.2 and 8.3). Twenty, 6-week old Swiss T.O. mice were each infected with one metacercarial cyst. These mice were necropsied at 10 days postinfection. Sixteen of the 20 mice became infected. Worms recovered from Generation 6 (see Chapter 8) of the multiple line were used as representatives of a cross-inseminated stock and compared with single infection line worms in each of the experimental procedures outlined in the following sections.

9.2.2 Isoenzyme analysis

An analysis of isoenzymes of worms from the multiple line (Generation 6) and single worms of Generation 4, was achieved by isoelectric focusing. The single infection line worms were from the re-established fourth generation described in section 9.2.1. In all, 21 Generation 6 worms were analysed and 11 single infection line worms Because of limited amounts of material, supernatants from each line were pooled and then run on electrofocusing gels.

Worms were dissected from mice at 10 days postinfection from both the multiple and single line and washed in 0.85% mammalian saline. These adult worms were then immediately stored in liquid nitrogen. Each worm was then macerated in an equal volume of distilled water and the mixture deep frozen for 1 hour before thawing and centrifuging at 49,000 g for 30 minutes at 4° C. The clear supernatants were used on the same day of preparation. The isoelectric focusing technique used is based on that of Karlsson, Davies, Ohman, Andersson (1973). The enzyme systems examined were malate dehydrogenase (MDH), phosphoglucomutase (PGM), acid phosphatase (AcP) and glucose phosphate isomerase (GPI). Polyacrylamide gels with a functional range pH from 4.5-9.5 were used for the separation of the enzyme systems studied. The gels were prepared from a basic formula of 72 ml of water in which 15 g of sucrose was dissolved and to which was added 20 ml each of 29.1% acrylamide and 0.9% N,N methylenebisacrylamide. Ampholine mixtures preblended by the manufacturer LKB were used with the gel mixture to produce the pH gradients using the

methods described by Ross (1976). The samples were placed at the anodal end of the gel using a pasteur pipette and human haemoglobin was used as a marker and applied anodally and cathodally. Electrofocusing was carried out using the LKB multiphor system. The plates were run for two hours for the completion of focusing and were maintained at 2-4°C during the run by circulating cooled ethylene glycol (75% aqueous solution) in the baseplate of the apparatus. The pH gradient of the focused gel was measured at intervals from the anode end edge of the plate using a Pye Ingold 8 mm membrane electrode (Ross, 1976). The separated enzymes were visualized using a 1% agar overlay incorparating the appropriate reagents (Ross,1976), the composition of these solutions are detailed by Wright, Southgate and Ross (1979). The isoelectric point (pI) for each fraction was determined by correlating the position of the band to pH gradient of the gel plate. The gel plates were then dried on a histological hotplate. Alongside the multiple line and single line worms of *E. liei*, samples of *Echinostoma togoensis* originating from Liberia that were maintained at the British Natural History Museum were prepared for isoelectric focusing to determine interspecific differences, if any, between *E. liei* and *E. togoensis*.

9.2.3 Light and electron microscopy studies

Single worms available from the original Generation 4 infection (see Section 8.2.2 and 8.3) and parallel single line infections (see Section 8.2.2.) were prepared for electron microscopy as described in Section 2.9.2. Additional worms from the single infection in section

9.2.1 were prepared for light microscopy using routine histological techniques and stained with haematoxylin and eosin (see Appendix 1). Worms from the Generation 4 re-infection in Section 9.2.1 were stained in aceto-orcein using the methods described in Section 2.8.4 to analyse the chromosomes of the testes. The paired testes of the worms were dissected out and squashed in aceto-orcein.

9.3 Results

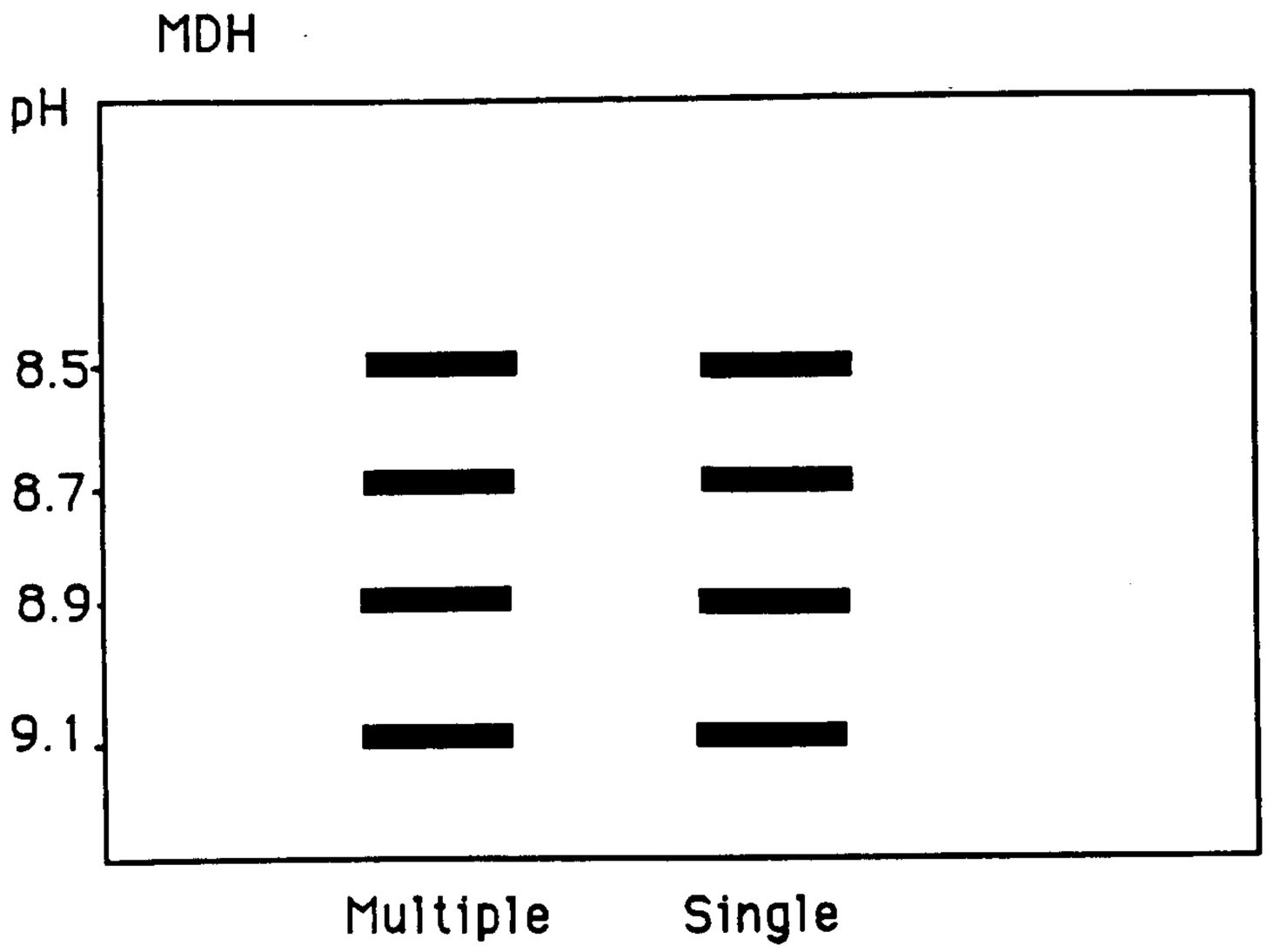
9.3.1 Isoelectric focusing of samples

The isoenzyme patterns obtained from the multiple line worms (A) and from the single line worms (B) of *E. liei* and the laboratory bred worms of *E. togoensis* (C) are shown in Figs. 9.1 and 9.2 alongside diagrammatic representations of the major bands from *E. liei* infections. The control sample human haemoglobin focuses at pH 7.25.

Malate dehydrogenase (MDH)

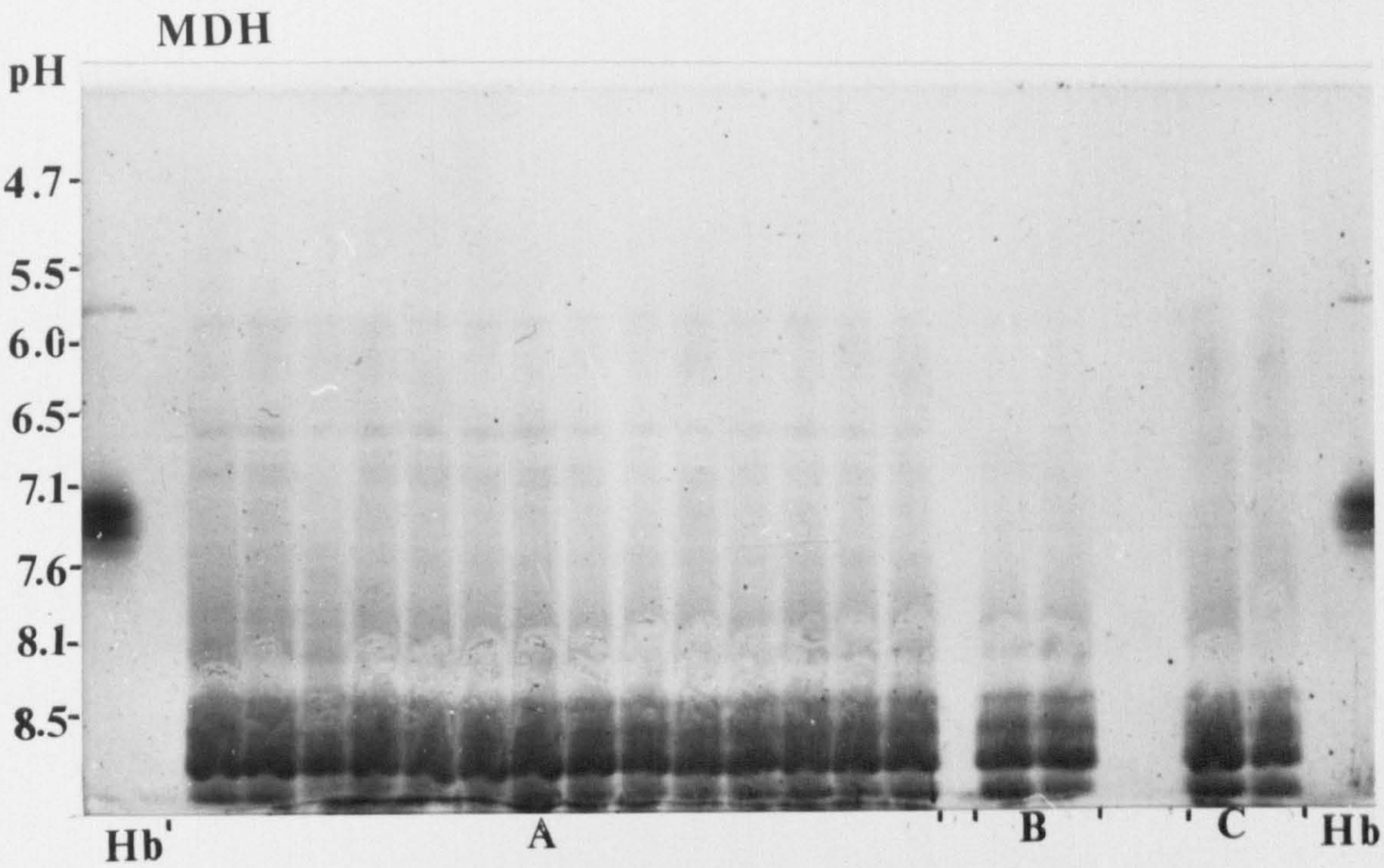
Patterns for both the multiple line and single line of *E. liei* and *E. togoensis* produce very strong bands with pIs 8.5, 8.7, 8.9 and 9.1. There is also in all three worm types a large number of much weaker bands within the range pI 6.0-8.5. There was no significant difference between the isoenzyme bands in the three worm types analysed.

Fig. 9.1 Major bands in E. liei



Key (see facing page)

A-E. liei (multiple) B-E. liei (single)
C-E. togoensis


MDH
pH
4.7
5.5
6.0
6.5
7.1
7.6
8.1
8.5
Hb
A
B
C
Hb

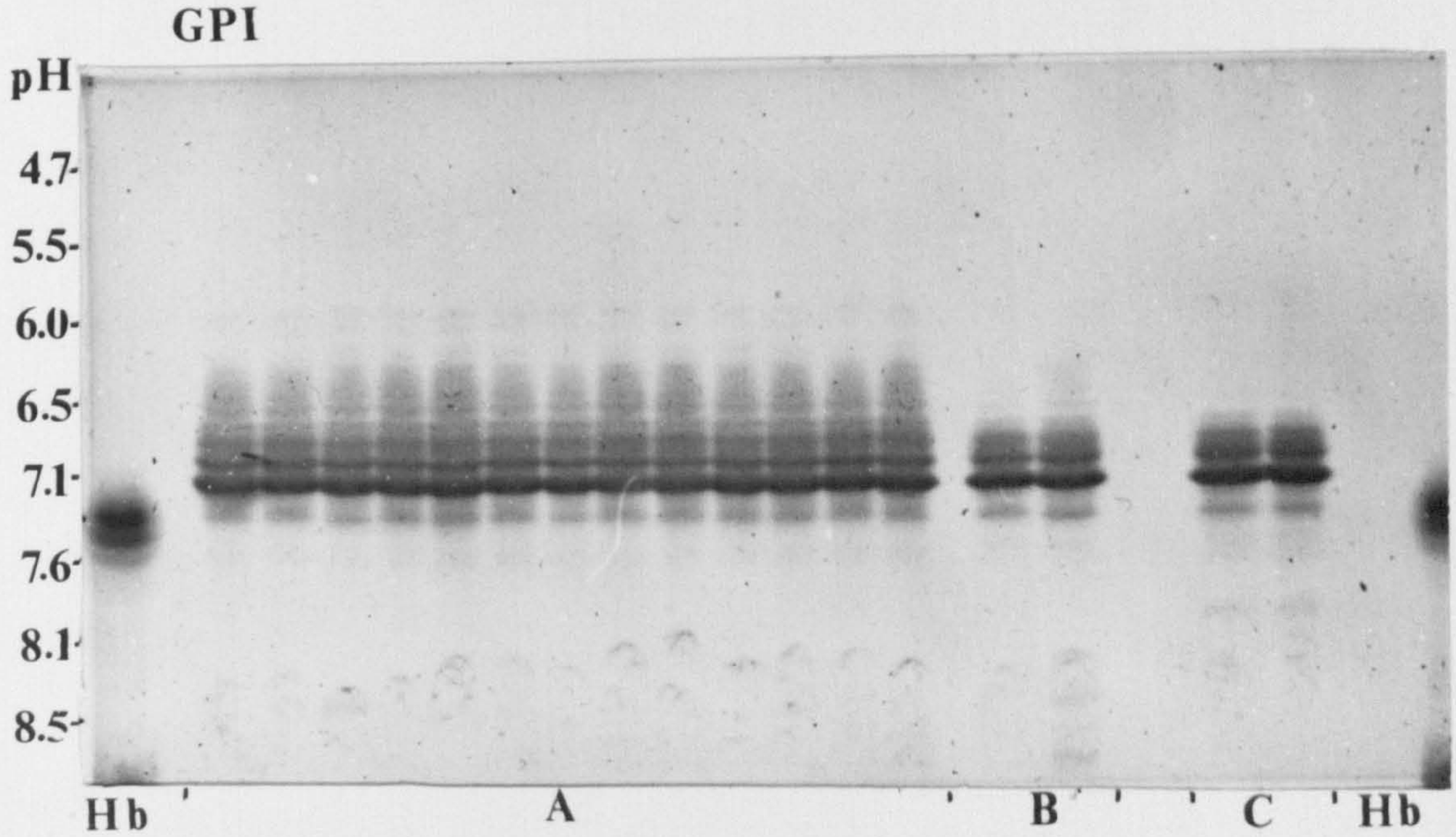
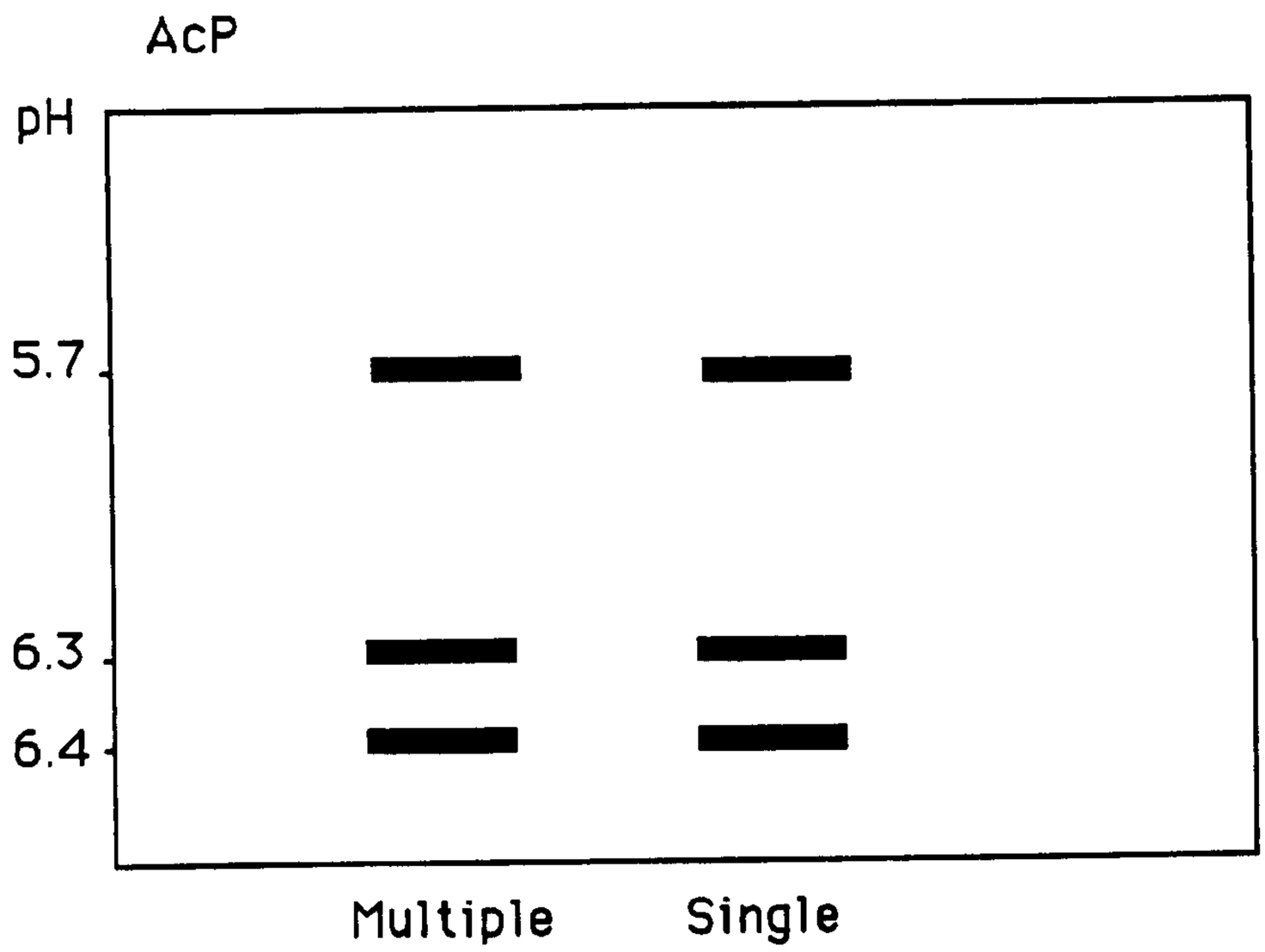
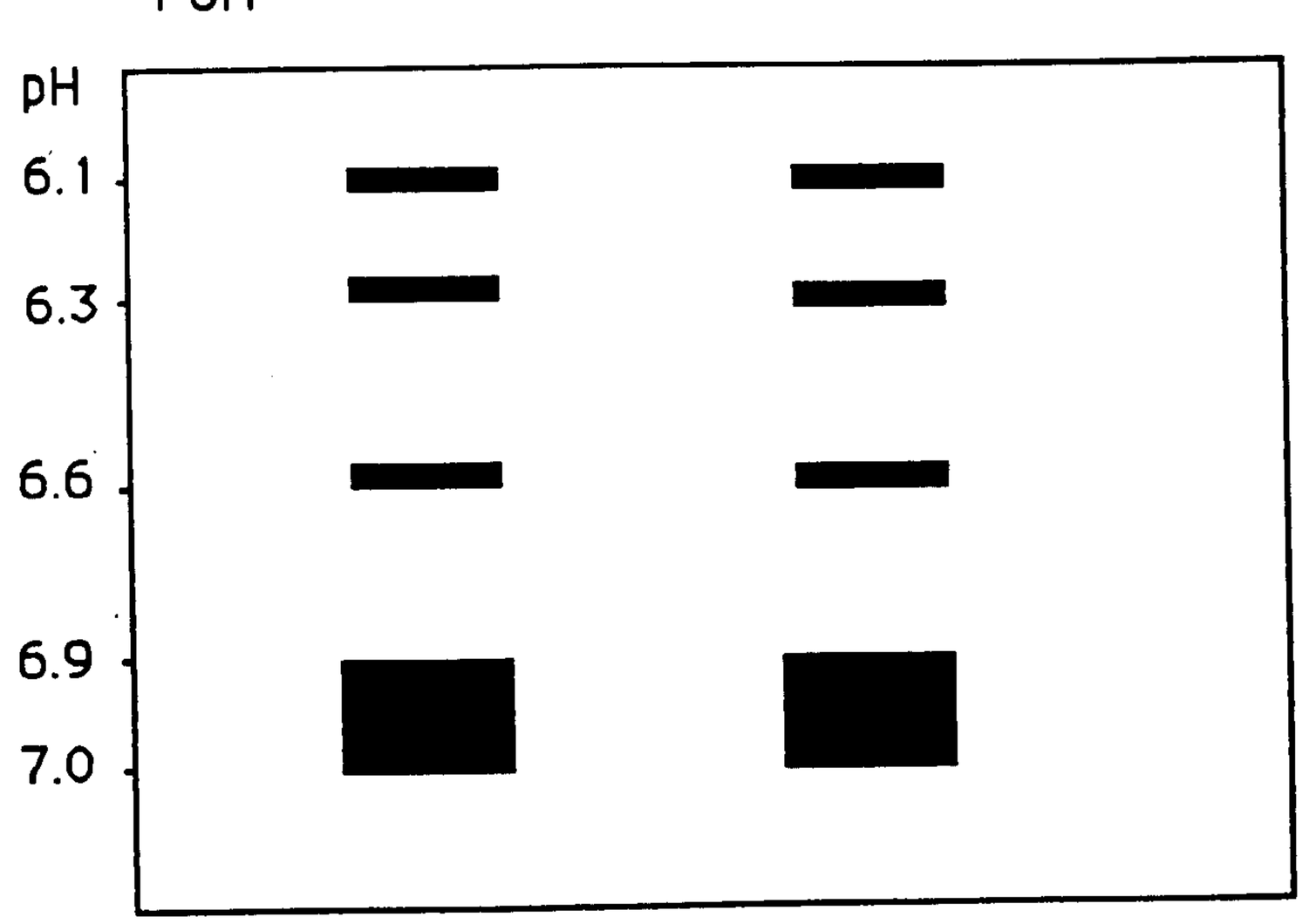

GPI
pH
4.7
5.5
6.0
6.5
7.1
7.6
8.1
8.5
Hb
A
B
C
Hb

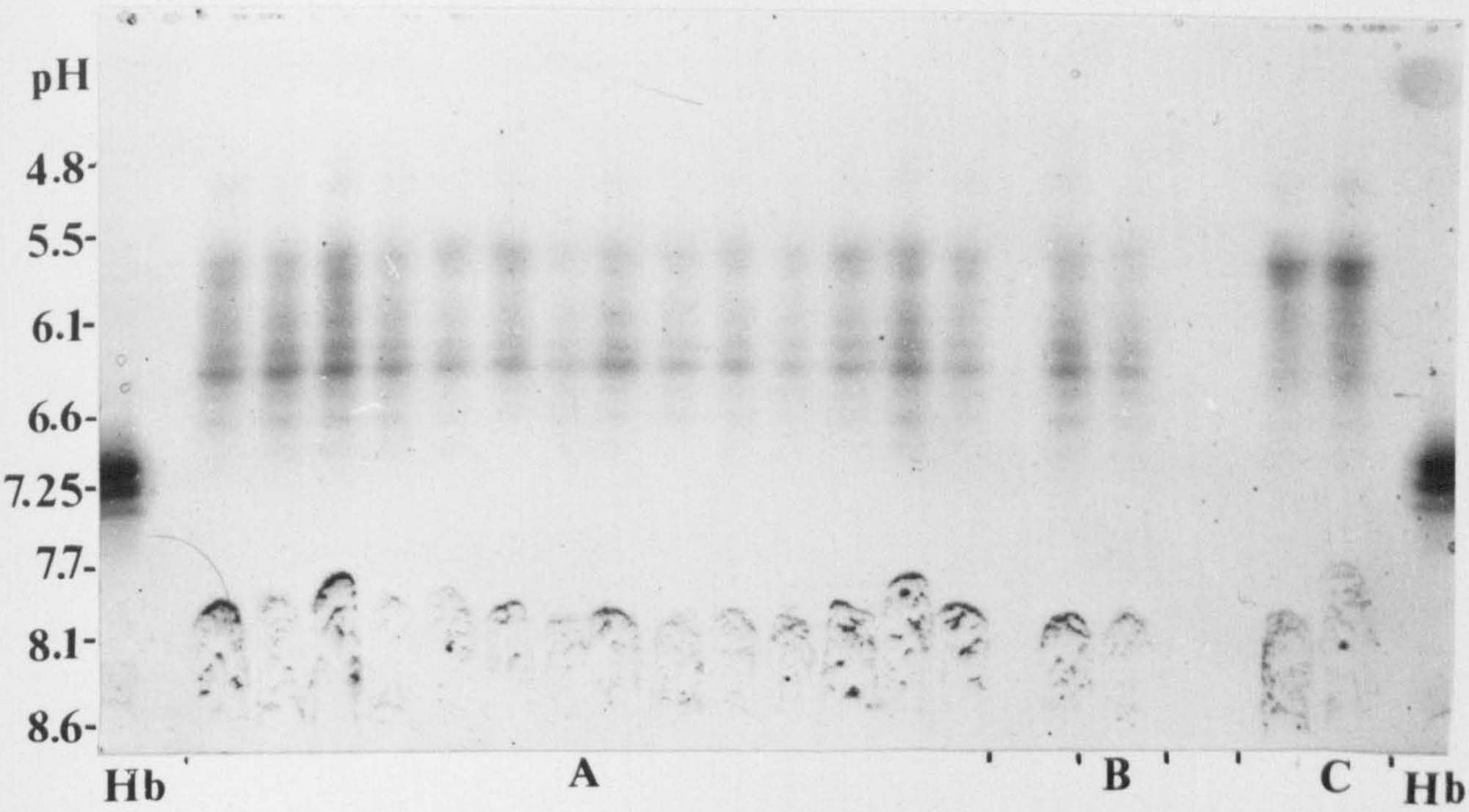
Fig. 9.2 Major bands in E. liei



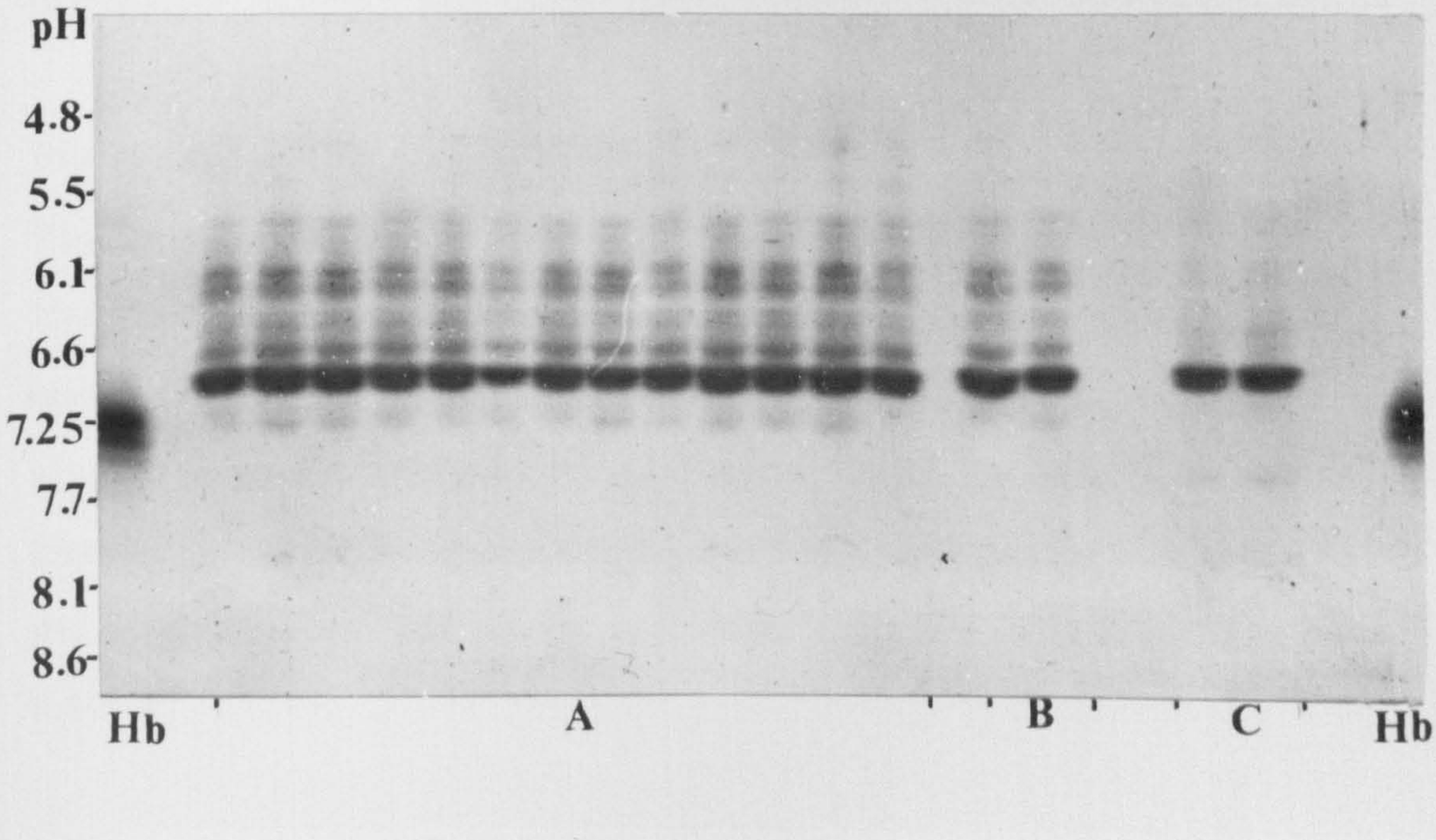


Key (see facing page)
A-E. liei (multiple) B-E. liei (single)
c-E. togoensis

AcP


pH
4.8
5.5
6.1
6.6
7.25
7.7
8.1
8.6
Hb
A
B
C
Hb

PGM


pH
4.8
5.5
6.1
6.6
7.25
7.7
8.1
8.6
Hb
A
B
C
Hb

Glucose phosphate isomerase (GPI)

With this enzyme, in all the worms studied, a moderate strength band is apparent with pI 7.0 and a very strong band at 7.1, weaker activity is seen at 6.7, 6.9 and 7.2. In the multiple line worms of *E. liei* minor bands of weaker activity are evident in the region 6.7-6.1. In both the single worms of *E. liei* and the worms of *E. togoensis* the activity in this region is very much weaker. Overall there are no differences in the major band patterns of the three worm types.

Acid phosphatase (AcP)

In both the multiple worms and single worms of *E. liei*, major bands separate with pIs 6.3, 6.4 and 5.7. There are also a whole range of faint and diffuse bands over the range 5.5 to 6.8. The major activity in *E. togoensis* is at 5.7 but faint activity exists at 6.3 and 6.4. Therefore in terms of major bands there are no significant differences between the three worm types.

Phosphoglucomutase (PGM)

The enzyme patterns in both the single and multiple worms of *E. liei* are identical. Common bands and major activity are apparent with pIs 6.6 and 6.9/7.0. Also strong but less intense bands are present at pI 6.1 and 6.3. *E. togoensis* shares a common major fraction in the PGM isoenzyme pattern with the worms of *E. liei* at pIs at 6.9/7.0 but appears to lack major fractions within the range 6.1-6.6.

9.3.2 Light microscopy

At the light microscopical level, major differences were observed in the sectioned reproductive anatomy of the single line worms of Generation 4 and the multiple worms of Generation 6. Fig. 9.3 (1) shows a longitudinal stained section of a single worm of *E. liei* from the fourth generation. From this low power view it can be seen that eggs are present in the uterus while the testes are grossly abnormal in comparison with sections showing the reproductive anatomy of worms from multiple infections as evident in Chapter 4 (see Fig. 4.2 (2) and (3)). In the Generation 4 single infection line worms, both of the testes are made up soley of large rounded cells in which meiosis appears to have been arrested (see Fig.9.3 (3)). No developing spermatozoa were present like those observed in the sections of worms from multiple infections (see Chapter 4, Fig. 4.2 (2)). The seminal vesicles contained no elongate sperm (Fig. 9.3 (2)) in marked contrast to worms from multiple infections (see Chapter 4, Fig. 4.2 (1)). Examination of further regions of these sections revealed no major differences between the two worm types. The ovary in these single worms appeared normal as did the surrounding somatic tissue.

9.3.3 Chromosome observations

A chromosome squash from the single line worms of *E. liei* is shown in Fig. 9.3 (4). It and squashes from the testes of multiple worm infections reveal that the haploid chromosome number of *E. liei* is 11 in both worm types. In squashes of the testes of multiple worms developing spermatozoa were observed. In the single worm

Fig. 9.3 (1-7)

(1) Section of fourth generation single worm

Scale bar=100 μm

(2) Cirrus pouch of a fourth generation single worm

Scale bar= 100 μm

(3) Section of testis of fourth generation single worm

Scale bar= 20 μm

(4) Chromosomes from fourth generation single worm

Scale bar= 5 μm

(5) Spermatocyte-like cells from fourth generation single worm

Scale bar= 2 μm

(6) Testis periphery from fourth generation single worm

Scale bar= 1 μm

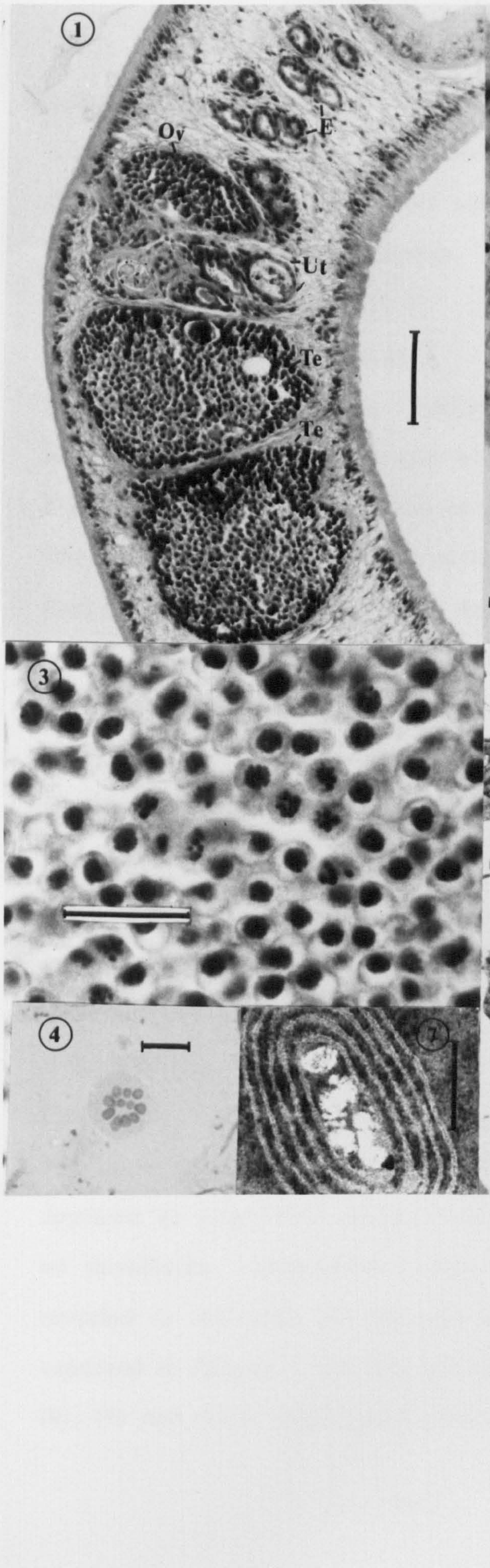
(7) Spermatocyte cell structures from fourth generation single worms

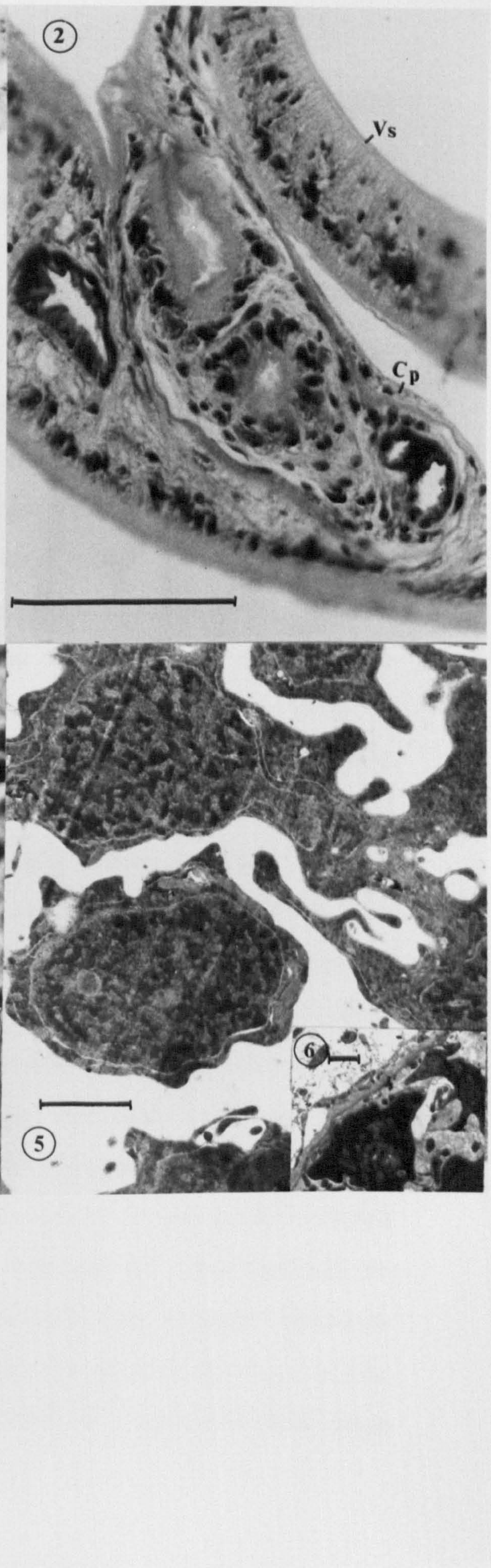
Scale bar= 0.5 μm

Key

Ov-ovary E-eggs Ut-uterus Te-testes

Vs-ventral sucker Cp-cirrus pouch


1
Ov
E
Ut
Te
Te


2
Vs
Cp

3

4

7

5

6

infections the chromosomes were identical to those of the multiple worm infections both in number and shape. In complete contrast to those of the multiple worms the squashes of the testes from single worms possessed no spermatozoa.

9.3.4 Electron microscopy

Ultrastructural observations highlighted the gross abnormality of development of testes of single worms from the fourth generation. Fig 9.3 (5) and (6) show views of the testes of these single worms. The testes appeared to be completely made up of very large, irregularly shaped, electron dense cells presumed to be spermatocytes. They possessed circular to oval-shaped nuclei which were filled with electron dense structures (Fig. 9.3 (7)). Some of these nuclei possessed chromatin arranged in clusters. The only recognisable structures within these cells were synaptonemal complexes (see Chapter 4, Fig. 4.4 (4)). These structures indicate that pairing of homologous chromosomes is occuring at the prophase stage in meiosis in some spermatocytes. The cytoplasm of these cells also appeared very dense and contained mitochondria (Fig. 9.3 (5)). The intercellular connections (cytophores) between these cells were very wide although some cells appeared to be connected to each other by tortuous cytoplasmic extensions that appeared as finger-like strands. Observations of the testes revealed no developing spermatozoa nor evidence of spermiogenesis as revealed in the adult 10 day old worms from multiple infections examined in Chapter 4 (see Fig. 4.4 (5) and (6); Fig. 4.5 (1), (2), (3), (8), (9) and (10)). High power observations of many of these large

irregularly shaped spermatocyte-like cells revealed few recognisable structures within them. Fig. 9.3 (7) reveals a structure present in the nucleus of these cells, which appears to consist of a membranous ovoid ring of 5 concentric bands, with electron-lucent structures at its centre. Fig. 9.3 (6) shows the membranous tissue layer which separates the somatic cells from the irregular shaped spermatocytes in the testes. These large spermatocyte-type cells filled the entire region of the testes of these 10 day old Generation 4 worms. Vitelline cell development was observed to be continuing normally as all the vitelline cell stages of vitellogenesis identified in Chapter 4 (see Section 4.3.2) were observed.

9.4 Discussion

In summary, observations on the offspring of a self-inseminating line of *E. liei* after four successive generations have revealed deleterious abnormalities in the organisation of the male regions of the reproductive organs of adult worms. This finding would appear to explain partially if not completely, the dramatic decline in the reproductive success demonstrated by the Generation 4 single line worms in Chapter 8. Eggs from these worms on the whole failed to develop and those that did develop, released miracidia, which failed to infect *B. glabrata* snails. This Chapter has proved unequivocally that the source of this problem was a characteristic of the worms themselves and not the result of external factors affecting miracidial hatching and infectivity. It can be presumed, then, that successive self-insemination, had given rise to a line of worms that lacked normal testes that could produce spermatozoa.

It is highly probable that those few eggs that did develop in the single line in Generation 4 did so parthenogenetically. This method of reproduction will be discussed further in Section 9.4.4. The following sections discuss the individual aspects of the results of this Chapter.

9.4.1 Genetic comparisons

Analyses of four enzymes of the multiple and Generation 4 single line worms of *E. liei* revealed no significant isoenzymes differences in PGM, GPI, AcP and MDH. Isoenzyme patterns of worms from both of these lines were similar in respect of major band locations although there were some differences in band intensity and in minor bands. These negative findings, that is, broad isoenzyme similarities between the single and multiple line worms must be treated with caution. Although no significant differences have been demonstrable in the four enzymes investigated, it is possible that a wider search using a larger range of enzymes might reveal isoenzyme differeneces that reflected genetic differences between the two lines.

Comparisons of the isoenzyme patterns of *E. liei* with those of the Liberian strain of *E. togoensis* also reveals that *E. liei* and *E. togoensis* show no major differences suggesting a close phylogenetic relationship between the two species. This appears to be in agreement with the hypothesis postulated by Voltz, Richard, Pesson and Jourdane (1988). These workers, using electrophoretic techniques applied to African Echinostomatidae, concluded that

four laboratory strains of *E. liei*, *E. togoensis*, *E. caproni* and *E. sp.* (Cameroon) have constant differences represented by the PGM and GPI isoenzymes. These workers interbred *E. liei* with *E. togoensis*, *E. caproni* and *E. sp.* to obtain F1-hybrids and to determine the allelic structures of both the parental strains and the F1-progeny. They found that 81% of the F2 progeny recovered had genotypes identical to that of *E. liei*. The remaining worms showed 3 combined genotypes differing from the parental laboratory strains and from the F1-hybrids. With the success these workers had in interbreeding *E. liei* with *E. caproni*, *E. togoensis* and *E.sp.* in obtaining a second generation of hybrids and in the common genotypic combinations which resulted, they speculated that the four strains were subspecies of a single species. The isoelectric focusing results demonstrated in this study would tend to support the hypothesis of Kanev (1985) that *E. liei* and *E. togoensis* are not different species but two geographical isolates.

9.4.2 Analysis of the chromosomes during gametogenesis

Squashes of the paired testes of worms from both the single and multiple line reveals that the haploid cell chromosome number is n=11. Analysis of worms from the fourth generation single worms revealed that there were no gross chromosomal abnormalities. The somatic karyotype of the proposed synonym of *E. liei*, *E. caproni*, was first documented by Richard and Voltz (1986) who demonstrated that *E. caproni* possessed 11 pairs of chromosomes. These workers after establishing the diploid chromosome number as 2n=22, outlined the morphology of some of the chromosomes

observed. They concluded that chromosomes 1, 2, 3 and 7 were acrocentric while chromosomes 4 and 5 were submetacentric. Previous work on the European strains of *Echinostoma revolutum* and *Echinostoma echinatum* has shown that the diploid cell's chromosome number based on redial and cercarial preparations is also 2n=22 (Muttaffova and Kanev, 1986). These workers determined that the 10 consecutive chromosome pairs are subtelocentric and the eleventh chromosome pair metacentric. Earlier work had established that both *Echinostoma barbosai* (Mutafova and Kanev, 1983) and *Echinostoma cinetorchis* (Terasaki, Moriyama, Tanis and Ishida, 1982) possessed 2n=22 chromosomes.

9.4.3 Abnormal development of the testes

Light and electron microscopy studies revealed that the testes of worms from the single line fourth generation were grossly deformed when compared with worms of the same age from multiple infections. The ultimate cause of the abnormal development of the testes in the single worms can only be speculated on, but is likely to be related to the cumulative effects of self-insemination with its presumed reduction in genetic variability and the increased chances of deleterious double recessive combinations arising.

Buttner (1955) was able to carry single infections of the digenean *Ratzia joyeuxi* through three successive generations with no apparent deleterious effects although the testes of the adult worms

were markedly atrophied. Buttner suggested that egg production in this situation may have arisen by parthenogenesis. Nollen and Alberico (1972) noticed a lack of testes in *Gorgoderina attenuata* recovered from frogs from field conditions but attributed this loss to poor nutrition of the host during winter. These workers stated that the testes of digeneans were the most sensitive organs to suboptimal conditions in culture media. Fujino and Ishii (1982) studied spermatogenesis in a parthenogenetic type of *Paragonimus westermani* which they termed *P. pulmonalis*. They noticed that during spermatogenesis most of the cells of the testes degenerated or appeared malformed. They observed normal spermatogonia at the periphery of the testis and spermatocytes at various development stages were distinguished. The presence of synaptonemal complexes led them to conclude that some pairing of homologous chromosomes was occurring at the prophase of meiosis in some spermatocytes. Some spermatocytes developed to spermatids and underwent nuclear elongation and flagella elongation. These workers very rarely observed profiles of spermatozoa in the testes and speculated that they may degenerate as none were observed in the seminal receptacle. The seminal receptacle, instead, contained eggs and vitelline cells. They observed many abnormal single cells, the most common having large membranous bodies with deformed organelles. Ribosomes or endoplasmic reticulum were never seen. Other abnormal cells contained numerous axial filaments, deformed mitochondria and glycogen arranged irregularly. These workers concluded that complete spermatogenesis in *P. pulmonalis* may have been

inhibited by aberrations resulting from a) disruption of syncytium formation, b) endopolyploidy in spermatogonia c) abnormalities in meiosis d) or the degeneration in cellular differentiation caused by the malfunctioning of cell organelles and protein synthesis. Some of these mechanisms may be applicable and operational in the Generation 4 single line worms of *E. liei*.

As haploid chromosome squashes have been made from such testes it is assumed that although meiosis is occurring the subsequent cytoplasmic alterations of spermiogenesis, the changing of a rounded haploid spermatid into an elongate flagellate spermatozoon, do not occur. If this is so, egg development can have only taken place in such worms parthenogenetically. Light microscopy of the ovary of *E. liei* in these worms indicated that the arrangement was similar to those worms recovered from multiple infections and electron microscopy identified normal vitelline cell development. This suggests that relatively normal oogenesis and vitellogenesis may be possible. If these result in unfertilised egg production, these may on some occasions develop parthenogenetically into miracidia.

Shaw (1987) witnessed the normal sequence of development and maturation of vitelline cells in single female infections of *S. mansoni* but noted development was limited to the posterior region of the worms. An incomplete form of parthenogenesis has been described by Taylor, Amin and Nelson (1969) in adult female single worm infections of *Schistosoma mattheei*. They found that in

these infections the unpaired worms matured and laid eggs that were non-viable. Similarly, Shaw (1987) concluded, after observing malformed eggs in single infections of *S. mansoni*, that whilst the male worm is not necessary to initiate vitelline gland development and egg production, it is essential for the complete maturation of the ovary. Shaw explained that within the genus *Schistosoma* the production of malformed or non-viable eggs by unisexual females is often termed incomplete parthenogenesis.

Basch and Basch (1984) have clearly demonstrated that female worms of *S. mansoni* are capable of parthenogenetic reproduction although in many cases both the somatic growth and development of the vitelline glands was less than in paired mature females while the onset of egg production was retarded and the number of eggs produced greatly reduced. In the hermaphroditic ectoparasitic digenean *Transversotrema*, in which single worm infections produce eggs at a normal rate, all eggs thus produced are non-viable (Whitfield, pers. comm.).

9.4.4 Benefits and costs of non-cross fertilising sexual reproduction in *E. liei*

Experimental passage of *E. liei* through the four generations of the single line results in the collapse of spermatogenesis and the subsequent disruption of reproductive success. This implies, at the very least that up to the point of the third generation in the single line, selfing must be occurring, that is, complete egg/sperm fusion. Presumably the later disruption of spermatogenesis is a result of a

genetic constitution formed by the fusion of eggs and sperm in the third generation single line. This new genotype has the consequence of producing a phenotype (after development) which has non-functional testes. In terms of the genetic constitution of these aberrant single line worms this alteration can be viewed in the following way. It can be speculated that the chances of a dangerous double recessive combination occurring increase statistically with every successive selfing generation. In this instance, this combination appears to have arisen after four generations and caused a failure of testis development. In another such parallel infection an equivalent dangerous combination might arise after a different number of generations and affect a quite different organ system or process. The collapse of spermatogenesis in the present experimental run exposes the low level of parthenogenetic development that can occur in this species.

It appears that *E. liei* can reproduce in single worm infections by having a capacity for selfing which in a single generation, achieves reproductive success levels similar to those generated by crossing. Coupled with this is, perhaps, a very low intrinsic capacity for parthenogenesis. In this experimental run, after four generations of selfing a presumed genetic perturbation produced a genotype that was unsuccessful because of a failure of spermatogenesis. The remaining parthenogenetic capacity was not sufficient to sustain the line in reproductive success terms. Once this presumed double recessive state had been reached, relaxing selfing conditions had little helpful effect, as all eggs and sperm would presumably

possess the dangerous recessive allele. This suggestion is supported by the results of the multiple infection utilizing metacercarial cysts derived from the single line fouth generation in Chapter 8. Eggs produced in this situation were non-viable. It can be presumed that reassortment and the mixing of these genotypes were unable to increase the chances of producing sexually successful worms.

Reproduction and the dissemination of the reproductive products are an esssential feature of the parasitic life cycle and reproduction often has to take account of low levels of infection (particularly the limiting case of one parasite per host) while fecundity levels must balance the large mortality faced at various stages of the life cycle (Kennedy, 1976). In bisexual infections, such as those of the dioecious schistosomes, in low density conditions the probability of mating may be very low (MacDonald, 1965). In the case of schistosomes, then, the extent of successful sexual mating depends on the size of the infection. On the whole, the majority of parasites with separate sexes minimize this problem because they are overdispersed within their host population (Crofton, 1971). This ensures that the aggregative dispersion of the parasites reduces the absolute number of instances where single worm infections occur.

From the evidence of the literature presented in Chapter 8 it can be seen that the phenomenon of successful self-fertilisation in hermaphroditic digeneans is apparently limited to a few species, while in most others the presence of another partner is essential for the production of viable eggs.

In those hermaphroditic digeneans such as *E. liei* that can successfully self-inseminate, this particular strategy is a clear advantage when single worm infections arise in the natural environment, hermaphoditism and selfing thus allowing successful multiplication to occur at very low population densities. Erwin and Halton (1983) have suggested that the elongation of the digenean spermatozoon and the reduction in its power of motility have increased the chances of internal fertilisation by reducing the chances of the spermatozoa swimming away from the ova. It is difficult to imagine selfing strategies being possible in helminths which did not utilize internal fertilisation. The overdispersed distribution of parasitic infections and the aggregation and clumping behaviour exhibited by *E. liei* in its final host would ensure that the probability of *E. liei* occurring in multiple infections where the conditions are available for cross-fertilisation would be very high.

Whitfield and Evans (1983), have pointed out that selfing will reduce the degree of genetic diversity and shuffling induced by sexual reproduction compared with that occurring in outbreeding dioecious forms. In the case of *E. liei,* these deficiencies have drastic consequences for the viability of the worms and therefore, if commonly met with in the natural environment could threaten the persistence of the parasite population in such circumstances.

According to Whitfield and Evans (1983), parthenogenesis within the platyhelminths is confined to a minority of adult cestodes and digeneans inhabiting their final hosts. Parthenogenetic reproduction has been described in certain cestodes such as *Diphyllobothrium erinacei* (Sasada, 1978) and is considered to occur in a number of caryophyllids (Mackiewicz, 1981). Digeneans exhibiting this particular mode of reproduction include both orthodox hermaphrodite species as *Fasciola sp.* (Sakaguchi, 1980) and *Paragonimius westermani* (Miyazaki, 1978; Fujino and Ishii, 1982) and the dioecious schistosome, *S. douthitti* (Short and Menzel, 1959). Short (1952) presented evidence for parthenogenesis in *Schistosomatium douthitti* after he found that, after two generations, unmated females produced eggs that were, on the whole, non-viable. Short attributed this loss of viability to chromosomal aberrations caused by lack of fertilisation. Interestingly, Short and Menzel (1959) commented on the lower infective capacity of miracidial populations produced parthenogenetically by single females of *S. douthitti* compared with those miracidia produced by bisexual infections. After exposure of snails to these parthenogenetically produced miracidia the chromosomal organisation of the cercarial embryos was examined. They speculated that the high proportion of haploid forms that were present were responsible for the low mean snail infectivity of the parthenogenetically produced miracidia. Short's (1952) work on single sex infections involving *S. douthitti* showed that male and female worms derived from uniparental miracidia could mature sexually into unisexual infections. He found that the female

worms laid eggs most of which appeared to be dead or degenerate. Interestingly, in the context of the results of this study, he noted that miracidia did develop in a small proportion of these eggs. Short concluded that these eggs had developed parthenogenetically as examination of stained whole mounts of these female worms revealed no spermatozoa. As pointed out by Price (1980) parthenogenesis offers certain benefits to ensure reproduction when the optimum requirements for normal reproduction are not present.

The parthogenetic production of eggs with viable miracidia from singly encysted metacercariae has been inferred as a means of development by MacFarlane (1939). Working with the digenean *Coitocaecum anaspidis,* MacFarlane failed to find sperm in the testes, seminal receptacle or seminal vesicle of any of the worms examined but found the ovary full of well formed eggs. He suggested that the miracidia resulting from these eggs were formed by parthenogenesis.

Whitfield and Evans (1983) pointed out that evidence for parthenogenetic development of eggs in parasitic platyhelminths had, on the whole, not been obtained by direct cytological observations of oogenesis but inferred from a lack of mature sperm and on the basis of aberrant spermatogenesis. Coupled with this was the fact that some worm taxa had been shown to possess a triploid karyotype. It should be emphasized that the tentative conclusion that pathenogenesis may occur in some cases in single

worm infections of *E. liei* is based on the type of indirect evidence described by Whitfield and Evans (1983).

Under natural conditions, it is assumed that the probability of an inbred line undergoing the extreme long term isolation adopted in this study is very low indeed. The close proximity, aggregation and pairing behavioural patterns of *E. liei* during their anterior migrations in the small intestine detailed in Chapter 5 would strongly suggest that cross-insemination is the normal reproductve behaviour of these worms. Fried and Wilson (1981b) have stated that *E. revolutum* emits pheromone that attract other adults of the same species and Fried (1986) has suggested that "worm pairing" (the coming together and contact of worms) may have certain reproductive advantages associated with it. In worms that are not site specific in the definitive host but depend on cross-insemination, difficulty would be encountered in pairing for copulation if there was no mechanism to ensure pairing and aggregation. This point is expanded by the observations of Miyazaki, Terasaki and Habe (1981) who observed the Japanese strain of *Paragonimus westermani* in single infections crawling in the pleural cavity without making the worm cyst. They concluded that the worms do not self-fertilise but have to exchange their sperms by pairing in the worm cyst and therefore single worms of *P. westermani* keep migrating until they meet a mate.

These workers produced unequivocal evidence that in *P. westermani* cross-fertilisation was indispensible. In *E. liei* the

importance of cross-fertilisation as a method of reproduction is broadly equivalent to that described for the Japanese strain of *P. westermani*. The main difference between the two species being that, in *E. liei*, several successive inbreeding cycles are required before reproductive success collapses. The probable chemoattraction and distinctive aggregation of worms of *E. liei* coupled with their migratory behaviour would ensure that the conditions for cross-fertilisation are maintained at a maximum level.

CHAPTER 10

GENERAL OVERVIEW AND DISCUSSION

General overview and discussion

This study, carried out on the echinostomatid digenean *E. liei*, has examined a range of biological facets relating to adult forms residing within a particular laboratory host, the Swiss T.O. mouse. The investigation's conclusions provide new knowledge about a member of the 37-spined echinostome group and in particular, one indigenous to Africa. Such information could be of utility in the design of any future integrated control programme that may seek to utilize a member of the African echinostomes as a biological control agent against human schistosomes. The knowledge gained also provides new insights into the capacity of gut-inhabiting digeneans to utilize self-fertilizing reproductive strategies as well as information about the development, growth and parthogenesis of such helminths. Finally, the results of this study can play a useful role in the continuing effort to unravel the complex systematic relationships between different 37-spined echinostomes (Christensen, Fried and Kanev, 1990; Huffman and Fried, 1990). The remainder of this general discussion addresses the main implications of the findings of particular Chapters in the thesis.

Information in both Chapter 3 and 4 has pointed to the suitability of Swiss T.O. mice as laboratory definitive hosts for the *E. liei* component of the *E. liei/caproni/togoensis* complex. Growth of somatic tissue and the development of the genitalia in the parasite proceeds in an orthodox and unhindered manner in this

mouse host. Ovigerous worms of *E. liei* were recovered at 8 days postinfection, a time period which coincided precisely with the completion of the development and functioning of the vitelline glands. As eggs were first observed both in the faeces and uteri of 8 day old worms it can be presumed that both the testes and ovary are also functional at this time. Fried and Emili (1987) conducted the first thorough examination of the growth of *E. liei* within the domestic chick and it is apparent from their findings, when compared with those from *E. liei* in Swiss T.O. mice, that body developmental patterns may differ markedly in different hosts. Chappell (1980) has pointed out that such differing growth patterns may involve a variety of physiological factors that include the immune response and the immunological status of the host and the physico-chemical conditions that promote or limit establishment and growth of a parasite. The growth and maturation of *E. liei* and the achievement of sexual maturity 8 days after initial infection in mice indicates the complete operational functioning of both spermatogenesis and oogenesis. Interestingly, the processes of vitellogenesis and spermatogenesis in *E. liei* bear a striking similarity to those of many other digeneans that have been studied, confirming the generalization of Hendow and James (1988) that these processes are characteristically similar in almost all digeneans at the ultrastructural level.

Microhabitat utilization by *E. liei* within the definitive mouse host is confined exclusively to the environment of the small intestine (see Chapter 5). Definite and distinctive ontogenetic migrations of this parasite take place, which move the worm populations in an anteriad direction towards the pyloric sphincter and away from the ileo-caecal valve. Such clear-cut anterior migrations are linked with the impressive aggregational behaviour of these worm populations. This study has conclusively shown, utilizing a simple near-neighbour analysis, that the mean distance between individual worms in a population decreases as the population ages. This phenomena manifests itself by the progressive aggregational behaviour of the worm populations. Fried and his co-workers have carried out numerous studies on the intraspecific and interspecific "pairing" of worms *in vitro* including studies involving *Echinostoma revolutum* (Fried and Jacobs, 1980; Fried, Tancer and Fleming, 1980; Fried and Wilson, 1981a,b; Fried and Pallone, 1984) which all indicate the ability of a number of worms to pair *in vitro* (in the sense of coming into close proximity). The present study has proved that a similar relationship and "pairing ability" occurs *in vivo*. Holmes (1973,1983) has concluded that the general phenomenon of site selection is co-evolved, driven by selection to avoid competition while Rhode (1979) has stated that site limitation may be the result of independent selection for greater contact for reproduction. The benefits of such a phenomenon can only be speculated on but as pointed out in Fried's (1986) conclusions on

the significance of close clusterings of worms, such patternings may provide nutritive, social and developmental advantages as well as reproductive ones.

The present study has shown that in *E. liei* infections, density-dependent constraints do enter into the host-parasite interplay with respect to egg production, affecting both the number of uterine eggs and the overall egg output per worm per day (see Chapter 6). Scott and Lewis (1987) have pointed out that a factor of central importance in determining the impact of density-dependent constraints is the manner in which the parasite population is distributed within the host population. They explained that given the normal pattern of over-dispersion it is clear that density-dependent constraints on parasite survival and reproduction will occur mainly in a few heavily infected hosts within the population. Ultimately, density-dependence in parasite survival and fecundity may arise as result of competition for limiting resources such as food and space as pointed out by Keymer (1982). In other echinostome host models, Odaibo, Christensen and Ukoli (1988) observed no density-dependent constraints on parasite fecundity in *E. caproni* while work outlined by Fried and Freeborne's observations on *E. revolutum* (1984) did demonstrate density-dependent constraints which they attributed to the phenomenon of worm crowding. Within the genus *Echinostoma* there is certainly evidence that parasite-induced host mortality is related to parasite density. Jourdane

and Kulo (1982) with *E. togoensis* have shown an initial metacercarial cyst density of 200 is capable of inducing the death of laboratory mouse hosts. Mortality in this instance is probably related to the physical carrying capacity of the aliamentary tract of the laboratory host and to the pathological and subsequent traumatic effects induced by such high density infections.

The pathology associated with *Echinostoma* infections has received considerable attention (Kishore and Sinha, 1982; Bindseil and Christensen, 1984; Huffman, Michos and Fried, 1986; Huffman, Alcaide and Fried, 1988). These observations, in general, mirror the pathology associated with *E. liei* infections outlined in Chapter 6. Taken together, such results signify that members of the genus *Echinostoma* have a marked pathological effect on the small intestine. In the case of *E. liei* parasitisation leads eventually to the erosion of the intestinal villi making the surface of the mucosa appear flat. Even at 8 days postinfection pathology is noticeable even in infections of low density, while in heavier infections the effects are more marked. The muscular hypertophy observed in mice infected with *E. liei* may aid the movement of food along the intestine during parasitisation as its development coincides with the dilation of the diameter of the intestine. Interestingly, in infections of the nematode *Trichostrongylus colubriformis*, two zones were delimited along the length of the parasitised mucosa in the rabbit host (Hoste,

Kerboeuf and Parodi, 1988). In the proximal part of the gut both shortened and dilated crypts were found while in the distal region (containing the lower density of parasites) dilation of the crypts was associated with an increase in size of the villi. Such an elongation of villi, these workers suggested, represented a compensatory hypertrophy as it was concomitant with the abrasion of the villi in the upper intestine. Given both the alteration in gut musculature mucosal architecture caused by *E. liei* infections it would be very interesting to compare the absorptive efficiency of mouse guts in control and *E. liei* infected states. The fact that *E. liei* can cause so much gut damage during infections would warrant considerable caution if schistosomiasis control programmes adopted the use of any of the 37-spined echinostomes. Echinostomes such as *E. liei* are able to utilize a wide range of both mammalian and avian species and therefore could be a potential pathological threat to a variety of species if the control programme aimed to increase echinostome population densities.

The demonstration of a vertebrate-like neuropeptide in *E. liei* associated with the outer tegument and its interconnected system of tegumentary cells is a novel finding (see Chapter 7 and Thorndyke and Whitfield, 1987). Such specific immunoreactivity of a sub-population of tegumentary cells has never before been established in any invertebrate system. The immunoreactivity of the sub-tegumentary cells appears to be linked to the somatic

development of the worms. Interestingly, no vertebrate VIP-like immunoreactivity was found in *Fasciola hepatica* (Magee, Fairweather, Johnston, Halton and Shaw, 1989) but was located in nerve fibres in *Diclidophora merlangi* (Maule, Halton, Johnston, Fairweather and Shaw, 1989). The possible role of a peptide such as VIP in the physical degradation of host tissue is intriguing and this area would warrant further investigation to enhance the present knowledge on the functioning of the host-parasite interface. The location and confinement of vertebrate VIP-like immunoreactivity in the outer tegument putatively points to a role connected with the intestinal environment of the host perhaps influencing musculature and blood vessels in the host gut wall. Such a physiological role may be an integral part of the relationship of *E. liei* with its vertebrate host. Recent confocal laser scanning techniques have provided the ability to observe the 3-dimensional distribution of peptides in whole mount preparations, thus providing a truer picture of their *in vivo* distribution. Further experimentation with the *E. liei*-mouse model could usefully involve such techniques. The study of neuropeptides in the *E. liei/caproni/togoensis* complex is evidently expanding with several recent new findings of significance. So, the neural and glandular localisation of substance P in *E. caproni* (Richard, Klein and Stoeckel, 1989) points to a neuropeptide production related to a developmental time sequence and a multi-hormonal capability in these echinostomes. The probable presence of a possible secretin-like material at a

specific time in a juvenile stage in *E. liei* (see Chapter 7) seems to be part of a similar developmental related pattern. A further addition to the *Echinostoma* peptide repertoire has been the demonstration and location of molluscan cardioactive tetrapeptide (FMRFamide)-like material, (a "native" invertebrate peptide) in *E. liei* (Riddell, Whitfield, Balogun, and Thorndyke, 1991). Distinct immunoreactivities have been located in cells and nerve tracts of the central nervous system of *E. liei* and also consistently within a solitary cell close to the terminal regions of the male parts of the reproductive tract. It is presumed that the FMRFamide-like material associated with the CNS may be utilized in peptidergic transmission related to muscular effector pathways. The FMRFamide-like material contained within the solitary cell of the cirrus may have a local neuromuscular association with muscles of the cirrus pouch. This peptide material may act as a neuromuscular transmitter which could have effects on the ejaculatory musculature and may even cause the eversion of the cirrus. Alternatively, the cell might release its products into the seminal fluids with effects on the musculature of the reproductive tract of a copulating partner after copulation, which may ensure the passage of sperm to the ootype.

The ability of *E. liei* to self-fertilise has been determined conclusively in Chapter 8. Such an ability has been previously attributed to certain other members of the genus *Echinostoma* (Fried, Huffman and Franco, 1988). In the limiting situation of one

worm of *E. liei* per host, self-fetilisation enables some offspring to be produced. The consequences of successive generations of self-fertilisation, however, are complex. At some point they appear to induce fatal deleterious effects on reproductive success. In the experiments described in Chapter 8 after 3 generation cycles of enforced self-fertilisation, the reproductive success of the self-fertilising line collapsed. The miracidia produced from eggs in the fourth self-fertilising generation were completely unable to infect snails. The results of the work described in Chapter 9 suggest that this deleterious change was the result of a complete inability of the Geneneration 4 worms to sustain normal spermatogenesis. The fact that such forms could produce eggs from which miracidia could hatch is presumed to have been the result of parthenogenesis. It is hypothesised that the acccumulation of damaging recessive alleles during successive self-fertilisation lead to the damaging loss of spermatogenesis.

In this context it is interesting to note that Fujino and Ishii (1982), working with a parthenogenetic type of *Paragonimus westermani*, also observed a complete lack of spematogenesis. In this lung fluke parthenogenetic reproduction was considered to be the norm. The findings relating to self-fertilising *E. liei* in the present study support one way in which such stably parthenogenetic forms as the *P. westermani* strain may be generated. If a genetic alteration due to self-fertilisation ablates spermatogenesis it will expose any residual parthenogenetic

capabilities. If these can sustain some gene flow to the next generation and there are concomitant selective advantages for the parthenogenetic state, the changed helminth might be able to sustain itself indefinitely as a purely parthenogenetic form.

In the case of *E. liei* at present, however, cross-fertilisation appears to be the normal and obligatory form of sexual reproduction over a period of many generations. The aggregational behaviour of these worms in their intestinal habitat outlined in Chapter 5 is likely to be of significance in enhancing the likelihood of such cross-fertilising encounters between worms. The present study offers novel insights into the long-term implications of self-fertilisation for the reproductive success of worms like *E. liei*.

The genetic comparison of *E. liei* and *E. togoensis* in Chapter 9 appears to imply that there are no major interspecific differences between the two in respect of the enzymes investigated. This observation would then tend to agree with the hypothesis of Kanev (1985), Christensen, Fried and Kanev (1990) and Huffman and Fried (1990), that is, that these *Echinostoma* species are geographical isolates of the same species. The biochemical results of Voltz, Richard and Pesson, (1987), Voltz, Richard, Pesson and Jourdane (1988) and Ross, Fried and Southgate (1989) are also in agreement with the views expressed by Kanev. It is envisaged that the future taxonomic organisation of this taxon of worms will

probably follow that of Kanev's original description since his work appears to clarify the biochemical studies that have been carried out. This is not to say that taxonomy should be based entirely on biochemical characterisation and in the process replace morphological observations. It should be noted that Kanev (1985) commented on the importance of morphology and used it in his revision of the *Echinostoma*, although he did point out that previous morphological features used to distinguish echinostomes were inappropriate. Morphology is still a very important source of taxonomic characters, as scanning electron microscope studies carried out in this study (see Chapter 3) and by Fried, Irwin and Lowry (1989) have demonstrated. SEM studies have revealed that it is possible to differentiate between the adults of *E. liei* and *E. revolutum* (American form) by the characteristic shape of the body spines of each (Fried, Irwin and Lowry, 1989). It is hoped that the SEM results of the present study will be of assistance in the resolution of the status of the components of the *E. liei/caproni/togoensis* complex. Once the status of these worms is resolved then work involving these echinostomes can continue unhindered by the complications that surround their taxonomy. It is evident, though, in this study that *E. liei* maintained in its mouse laboratory host, is a very suitable model to study aspects of the biology of the adult worms.

Literature cited

Andersen, K., Simonsen, P.E., Andersen, B.J. & Birch-Andersen, A. (1989). *Echinostoma caproni* in mice : shedding of antigens from the surface of an intestinal trematode. *International Journal for Parasitology* **19**, 111-118.

Anderson, R.M. (1978). The regulation of host population growth by parasitic species. *Parasitology* **76**, 119-157.

Anderson, R.M. & May, R.M. (1985). Helminth infections of humans : mathematical models population dynamics and control. *Advances in Parasitology* **24**, 1-101.

Anderson, R. M. & May, R.M. (1978). Regulation and stability of host-parasite population interactions. I. Regulatory processess. *Journal of Animal Ecology* **47**, 219-248.

Anderson, R.M. & Schad, G.A. (1985). Hookworm burdens and faecal egg counts : an analysis of the biological basis of variation. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **79**, 812-825.

Armstrong, J.C. (1965). Mating behaviour and development of schistosomes in the mouse. *The Journal of Parasitology* **51**, 605-616.

Bacha, W.J. (1966). Viable egg production in *Zygocotyle lunata* following monometacercarial infections. *The Journal of Parasitology* **52**, 1216-1217.

Bakke, T.A. (1976). Functional morphology and surface topography of *Leucochloridium* sp (Digenea), revealed by scanning electron microscopy. *Zeitschrift fur Parasitenkunde* **51**, 115-128.

Barrett, J., Cain, G.D. & Fairbairn, D. (1970). Sterols in *Ascaris lumbricoides* (Nematoda), *Macracanthorhynchus hirudinaceus* and *Moniliformis dubius* (acanthocephala) and *Echinostoma revolutum* (Trematoda). *The Journal of Parasitology* **56**, 1004-1008.

Basch, P.F. & Basch, N. (1984). Intergenic reproductive stimulation and parthenogenesis in *Schistosoma mansoni*. *Parasitology* **89**, 369-376.

Bayer, F.A.H. (1954). Larval trematodes from some freshwater snails. A suggested biological control method for bilharzia control. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **48**, 414-418.

Beaver, P.C. (1937). Experimental studies on *Echinostoma revolutum* (Froelich) a fluke from birds and mammals. *Illinois Biological Monographs* **15**, 1-96.

Beaver, P.C. (1980). Parasite factors influencing pathogenicity, mortality and morbidity of human intestinal infections. WHO scientific workshop group on intestinal protozoan and helminthic infections. *International Journal for Parasitology* SG/WP/80.3.

Bell, E.J. & Smyth, S.D. (1958). Cytological and histochemical criteria for evaluating development of trematodes and pseudophyllidean cestodes *in vivo* and *vitro*. *Parasitology* **43**, 131-148.

Bennett, C.E. (1975). Scanning electron microscopy of *Fasciola hepatica* L. during growth and migration in the mouse. *The Journal of Parasitology* **61**, 892-898.

Bennett, C.E. & Threadgold, L.T. (1973). Electron microscope studies of *Fasciola hepatica* XIII. Fine structure of newly excysted juvenile. *Experimental Parasitology* **34**, 85-89.

Bennett, C.E. & Threadgold, L.T. (1975). *Fasciola hepatica* : Development of tegument during migration in the mouse. *Experimental Parasitology* **38**, 38-55.

Bennett, S. & Fried, B. (1983). Densitometric thin-layer chromatographic analyses of free sterols in *Echinostoma revolutum* (Trematoda) adults and the excretory-secretory products. *The Journal of Parasitology* **69**, 789-790.

Berntzen, A.K. & Macy, R.W. (1969). *In vitro* cultivation of *Sphaeridiotrema globulus* (Rudolph) from the metacercarial stage to egg production. *The Journal of Parasitology* **55**, 136-139.

Berntzen, H. K. & Mueller, J.F. (1972). *In vitro* cultivation of *Spirometra* spp. (Cestoda) from the plerocercoid to gravid adult. *The Journal of Parasitology* **58**, 750-752.

Bindseil, E. & Christensen, N.O. (1984). Thymus-independent hyperplasia and villous atrophy in the small intestine of mice infected with the trematode *Echinostoma revolutum*. *Parasitology* **88**, 431-438.

Bishop, A.E., Polak, J.M. & Bloom, S.R. (1981). Pathology of gut peptidergic innervation. In 'Gut hormones' S.R. Bloom and J.M. Polak editors. Livingstone, Edinburgh.

Bjorkman, N. & Thorsell, W. (1963). On the fine morphology of the formation of egg shell globules in the vitelline gland of the liver fluke, *Fasciola hepatica* L. *Experimental Cell Research* **32**, 153-156.

Bjorkman, N. & Thorsell, W. (1964). On the fine structure and resorptive function of the cuticle of the liver fluke *Fasciola hepatica* L. *Experimental Cell Research* **33**, 319-329.

Bloom, S.R. (1977). Gastrointestinal hormones In : International Review of Physiology, Gastrointestinal Physiology II. 12, ed. R.K. Crane University Park Press. Baltimore.

Bloom, S.R. & Polak, J.M. (1978). Gut hormone overview In 'Gut hormones' (S.R. Bloom Ed.) Livingstone Edinburgh.

Bogitish, B.J. (1971). Golgi complexes in the tegument of *Haematoloechus medioplexus*. *The Journal of Parasitology* **57**, 1373-1374.

Bogitsh, B.J. (1968). Cytochemical and ultrastructrural observations on the tegument of the trematode *Megalodiscus temperatus*. *Transactions of the American Microscopical Society* **87**, 477-486.

Bone, I.W. (1982). Reproductive chemical communication of helminths. I. Platyhelminths. *International Journal of Invertebrate Reproduction* **5**, 261-268.

Boray, J.C. (1969). Experimental fascioliasis. *Advances in Parasitology* **7**, 95-210.

Boray, J.C. (1959). Studies on intestinal amphistomiasis in cattle. *Australian Veterinary Journal* **35**, 282-287

Bradley, D.J. (1972). Regulation of parasite populations. A general theory of the epidemiology and control of parasitic infections. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **66**, 697-708.

Braten, T. & Hopkins, C.A. (1969). The migrations of *Hymenolepis diminuta* in the rats intestine during normal development and following surgical transplantation. *Parasitology* **59**, 891-905.

Breckenridge, W.R. & Nathanael, S. (1988). Vitelline cell histochemistry in the commensal temnocephalid *Paracaridinicola platei* (Fernando, 1952) Baer, 1953, together with some notes on the egg. *Journal of Helminthology* **62**, 167-174.

Buffa, R., Capella, C., Solcia, E., Fergeria, B. & Said, S.I. (1977). Vasoactive intestinal peptide (VIP) cells in the pancreas and gastrointestinal mucosa. *Histochemistry* **50**, 217-227.

Burton, P.R. (1972). Fine structure of the reproductive system of a frog lung fluke. III. The spermatozoon and its differentiation. *The Journal of Parasitology* **58**, 68-83.

Burton, P.R. & Silveira, M. (1971). Electron microscopical and optical diffraction studies of negatively stained axial units of certain platyhelminth sperm. *Journal of Ultrastructural Research* **36**, 757-767.

Buttner, A. (1955). Les distomes progenetiques sont-ils des pre-adults au des adulles veritables? Baleur evolutive de la progenene chez les Digenea. *Comptes Rendus Societe de Biologie* **149**, 267-272.

Cambell, R.C. (1979). Statistics for biologists 2nd ed. Cambridge University Press.

Castro, G.A. (1982). Immunological regulation of epithelial function. *American Journal of Physiology* **243**, 9321-9329.

Chappell, L.H. (1980). Physiology of parasites. Blackie : Glasgow and London.

Cheever, A.W., Kamel, I.A., Elwi, A.M. Mosimann, J.E. & Danner, R. (1977). *Schistosoma mansoni* and *S. haematobium* infections in Egypt II. Quantitative parasitological findings at necropsy. *American Journal of Tropical Medicine and Hygiene* **26**, 702-716.

Chey, W.Y., Rhodes, R.A. & Tai, H.H. (1978). Role of secretin in man II. In : 'Gut hormones' S.R. Bloom ed. Livingstone, Edinburgh.

Christensen N.O., Fransden, F. & Roushdy, M.Z. (1980). The influence of environmental conditions and parasite intermediate host related factors on transmission of *Echinostoma liei*. *Zeitschrift fur Parasitenkunde* **63**, 47-63

Christensen, N.O., Fried, B. & Kanev, I. (1990). Taxonomy of 37-collar spined *Echinostoma* (Trematoda : Echinostomatidae) in studies on the population regulation in experimental rodent hosts. *Angewendte Parasitologie* **31**, 127-130.

Christensen, N.O., Odaibo, A.B. & Simonsen, P.E. (1988). *Echinostoma* population regulation in experimental rodent definitive hosts. *Parasitology Research* **75**, 83-87.

Colgan, G.J. & Nollen, P.M. (1977). Studies on the growth, development and infectivity of *Philophthalmus hegeneri* Penner and Fried 1963 in the chick. *The Journal of Parasitology* **63**, 675-680.

Combes, C. (1982). Trematodes: Antagonism between species and sterilizing effect on snails in biological control. *Symposia of the British Society for Parasitology* **19**, 151-175

Cox, D.D., Ciordia, H. & Jones, W.W. (1956). Variations of *Hymenolepis serrula* Oswald 1951 (Cestoda: Hymenolepidae) a cestode from the smoky shrew, *Sorex fumeus* Millon 1895. With special reference to three geographic areas. *Journal of the Tennessee Academy of Sciences* **31**, 289-299.

Crofton, H.D. (1971). A quantitative approach to parasitism. *Parasitology* **62**, 179-193.

Croll, N.A., Anderson, R.M., Gyorkos, T.W. & Ghardirian, E. (1982). The population biology and control of *Ascaris lumbricoides* in a rural community in Iran. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **76**, 187-197.

Crompton, D.W.T. (1973). The sites occuppied by some parasitic helminths in the aliamentary tract of vertebrates. *Biological Reviews* **47**, 27-83.

Crompton, D.W.T. & Whitfield, P.J. (1968). A hypothesis to account for the anterior migrations of adult *Hymenolepis diminuta* (Cestoda) and *Moniliformis dubius* (Acanthocephala) in the intestine of rats. *Parasitology* **58**, 227-229.

Cross, J.H. & Basaca-Sevilla, V. (1986). Studies on *Echinostoma ilocanum* in the Phillippines. *Southeast Asian Journal of Tropical Medicine and Public Health* **17**, 23-27.

Dacie, .J.V. & Lewis, S.M. (1984). Practical Haematology. Sixth Edition Churchhill-Livingstone.

Danley, D.L. (1973). The Harderian gland and site finding by metacercariae of *Philophthalmus megalurus* (Trematoda : Digenea). *The Journal of Parasitology* **59**, 457-462.

Davenport, T.R.B. Lee, D. & Issac, R.E., (1988). Immunocytochemical demonstration of a neuropetide in *Ascaris suum* (Nematoda) using an antiserum to FMRF-amide. *Parasitology* **17**, 81-88.

Davies, C. & Smyth, J.D. (1978). *In vitro* cultivation of *Fasciola hepatica* metacercariae and of partially developed flukes recovered from mice. *International Journal for Parasitology* **8**, 125-131.

Dawes, B. (1963). Hyperplasia of the bile duct in fascioliasis and its relation to the problem of nutrition in the liver fluke *Fasciola hepatica* L. *Parasitology* **53**, 123-133.

Dawes, B. (1962). On the growth and maturation of *Fasciola hepatica* L, in the mouse. *Journal of Helminthology* **36**, 11-38.

Demian, E.S. & Kamel, E.G. (1972). Growth and population dynamics of *Bulinus truncatus* under semi-field conditions in Egypt. *Proceedings of the Egyptian Academy of Science* **25**, 37-60.

Dockray, G.J. (1977). Molecular evolution of gut hormones : application of comparative studies on the regulation of digestion. *Gastroenterology* **72**, 344-358.

Domenici, L. & Gremigni, V. (1974). Electron microscopical and cytochemical study of vitelline cells in the freshwater triclad *Dugesia lugubris* s. I. II. Origin and distributiuon of reserve materials. *Cell and Tissue Research* **152**, 219-228.

Ebeid, A.M., Escurrou, J., Murray, P. & Fischer, J.E. (1978). Pathophysiology of VIP. In 'Gut hormones' S.R. Bloom, ed. Livingstone, Edinburgh.

El Emam, M.A. & Madsen, H. (1982). The effect of temperature, darkness, starvation and various food types on growth, survival and reproduction of *Helisoma duryi*, *Biomphalaria alexandrina* and *Bulinus truncatus* (Gastropoda: Planorbidae). *Hydrobiologica* **88**, 265-275.

Elliot, J.M. (1983). Some methods for the statistical analysis of samples of benthic invertebrates. Second edition. *Freshwater Biological Association Scientific Publications* no. **25**.

Erasmus, D.A. (1967). The host parasite interface of *Cyathocotyle bushiensis* Khan 1962 (Trematoda : Strigeoidea) II. Electron microscope studies of the tegument. *The Journal of Parasitology* **53**, 703-714.

Erspamer, V. (1984). Half a century of comparative research on biogenic amines and active peptides in amphibian skin and molluscan tisuues. *Comparative Biochemistry and Physiology* **79C**, 1-7.

Erwin, B.E. & Halton, D.W. (1983). Fine structural observations on spermatogenesis in a progenetic trematode *Bucephaloides gracilescens*. *International Journal for Parasitology* **13**, 413-426.

Evans, D.F., Pye, G., Bramley, R., Clark, A.G., Diynom, T.J. & Hardcastle, J.D. (1988). Measurment of gastrointestinal pH profiles in normal and ambulant human subjects. *Gut* **29**, 1035-1041.

Evans, N.A. (1983). The establishment and survival of *Echinoparyphium recurvatum* in the definitive host. *Zeitschrift fur Parasitenkunde* **69**, 401-402.

Evans, N.A. (1985). The influence of environmental temperature upon the transmission of the cercariae of *Echinostoma liei* (Digenea: Echinostomatidae). *Parasitology* **90**, 269-275

Fairweather, I., MaCartney, G.A., Johnston, C.F., Halton, D.W. & Buchanan, K.D. (1988). Immunocytochemical demonstration of 5-hydroxytryptamine (serotonin) in the nervous system of the liver fluke, *Fasciola hepatica* (Trematoda, Digenea). *Parasitology Research* **73**, 755-758.

Falkner, S., Hakansen, R. & Sundler, F. (1984). 'Evolution and tumour pathology of the neuroendocrine system' Elsevier, Amsterdam.

Fan, P.C. & Chang, C.H. (1970). Exposure of kittens and puppies to single metacercariae of *Paragonimus westermani* from Taiwan. *The Journal of Parasitology* **56**, 48-54.

Faust, E.C., Russell, P.F. & Jung, R.C. (1970). Craig and Faust's Clinincal Parasitology. Philadelphia : Lea & Febiger.

Ferguson, A. & Jarrett, E.E. (1975). Hypersensitivity reactions in the small intestine. I. Thymus dependence of experimental partial villous atrophy. *Gut* **16**, 114-117.

Fischthal, J.H., Carson, D.O. & Vaught, R.S. (1982). Comparative size allometry of the digenetic trematode *Lissorchis attenuatus* (Monorchidae) at four intensities of infection in the white sucker. *The Journal of Parasitology* **68**, 314-318.

Flavell, D.J., Flavell, S.V. & Field, G.F. (1983). *Opisthorchis viverrini* : The relationship between egg production, worm size and intensity of infection in the hamster. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **77**, 538-545.

Foreyt, W.J. Samuel, W.M. & Todd, A.C. (1977). *Fascioloides magna* in white-tailed deer (*Odocoileus virginianus*) : observations on the pairing tendency. *The Journal of Parasitology* **63**, 1050-1052

Foreyt, W.J. & Todd, A.C. (1976). Development of the large American liver fuke, *Fascioloides magna* in white tailed deer, cattle and sheep. *The Journal of Parasitology* **62**, 26-32.

Franco, J., Huffman, J.E. & Fried, B. (1986). Infectivity, growth and development of *Echinostoma revolutum* (Digenea :

Echinostomatidae) in the golden hamster, *Mesocricetus auratus*. *The Journal of Parasitology* **72**, 142-147.

Franco, J., Huffman, J.E. & Fried, B. (1988). The effects of crowding on the adults of *Echinostoma revolutum* (Digenea: Echinostomatidae) in experimentally infected golden hamsters *Mesocricetus auratus*. *The Journal of Parasitology* **74**, 240-243.

Fried, B. (1986). Chemical communication in hermaphroditic digenetic trematodes. *Journal of Chemical Ecology* **12**, 1659-1677.

Fried, B. (1984). Infectivity, growth and development of *Echinostoma revolutum* (Trematoda) in the domestic chick. *Journal of Helminthology* **58**, 241-244.

Fried, B. (1962). Growth of *Philophthalmus* sp. (Trematoda) in the eyes of chicks. *The Journal of Parasitology* **48**, 395-399.

Fried, B. (1985). Histochemical localization of alkaline phosphatase activity in *Leucochloridiomorpha constantiae* (Trematoda) cultivated on the chick chorioallantois. *The Journal of Parasitology* **71**, 510-512.

Fried, B. & Alenick, D.S. (1981). Localisation, length and reproduction in single and multiple worm infections of *Echinostoma revolutum* (Trematoda) in the chick. *Parasitology* **82**, 49-53

Fried, B. & Bradford, J.D. (1984). Site-finding of *Leucochloridiomorpha constantiae* (Trematoda) metacercariae to the bursa of Fabricius of the domestic chick. *The Journal of Parasitology* **70**, 308-309.

Fried, B & Caruso J.H. (1970). Histological observations on the intestine of *Echinostoma revolutum* and its ingesta. *Transactions of the American Microscopical Society* **89**, 110-112.

Fried, B. & Diaz, V. (1987). Site-finding and pairing of *Echinostoma revolutum* (Trematoda) on the chick chorioallantois. *The Journal of Parasitology* **73**, 546-548.

Fried, B., Donovick, R.A. & Emili, S. (1988). Infectivity, growth and development of *Echinostoma liei* (Trematoda) in the domestic chick. *International Journal for Parasitology* **18**, 413-414.

Fried, B. & Emili, S. (1987). Comparative studies on infectiviy, growth, excystation and cultivation of *Echinostoma revolutum* and *E. liei* (Trematoda). First International Autumn School, Parasite Host Environment, Varna, Bulgaria, p.193-206.

Fried, B. & Emili, S. (1988). Excystation *in vitro* of *Echinostoma liei* and *E. revolutum* (Trematoda) metacercariae. *The Journal of Parasitology* **74**, 98-102.

Fried, B., Fine, R.H. & Felter, B.L. (1980). Growth development and pairing of *Leucochloridiomorpha constantiae* (Trematoda) metacercariae on the chlorioallantois of chick embryos cultivated *in vitro*. *Parasitology* **81**, 41-45.

Fried, B. & Freeborne, N.E. (1984). Effects of *Echinostoma revolutum* (Trematoda) adults on various dimensions of the chicken intestine, and observations on worm crowding. *Proceedings of the Helminthological Society of Washington* **51**, 297-300.

Fried, B. & Fujino, T. (1984). Scanning electron microscopy of *Echinostoma revolutum* (Trematoda) during development in the chick embryo and the domestic chick. *International Journal for Parasitology* **14**, 75-81.

Fried, B. & Gioscia, R.M. (1976). Tentative identification of cholesterol as a chemoattractant for metacercraial pairing of *Leucochloridiomorpha constantiae* (Trematoda). *The Journal of Parasitology* **62**, 326-327.

Fried, B & Harris, K.R. (1971). Reproduction in single and double worm infections of *Leucochloridiomorpha constantiae* (Mueller, 1935) Trematoda in the Chick. *The Journal of Parasitology* **57**, 866-868.

Fried, B., Huffman, J.E. & Franco, J. (1988). Single and five worm infections of *Echinostoma revolutum* (Trematoda) in the golden hamster. *International Journal for Parasitology* **18**, 179-181.

Fried, B., Huffman, J.E. & Weiss, P.M. (1990). Single and multiple worm infections of *Echinostoma caproni* (Trematoda) in the golden hamster. *Journal of Helminthology* **64**, 75-78.

Fried, B., Irwin, S.W.B. & Lowry, S. (1989). Scanning electron microscopy of *Echinostoma revolutum* and *E. liei* adults. Abstracts of the British Society for Parasitoology Spring Meeting, Southampton University p. 38.

Fried, B. & Jacobs, J.E. (1980). Pairing between *Echinostoma revolutum* (Trematoda) adults and other hermaphroditic digeneans

in vitro. *Proceedings of the Helmintholofgical Society of Washington* **47**, 136-138.

Fried, B. & Kletkewicz, K. (1987). Excystation of *Echinostoma revolutum* in the domestic chick. *Proceedings of the Helminthological Society of Washington* **54**, 267-268.

Fried, B. & Morrone, L.J. (1970). Histochemical lipid studies on *Echinostoma revolutum*. *Proceedings of the Helminthological Society of Washington*, **37**, 122-123.

Fried, B. & Pallone, A. (1984). Further studies on *in vitro* pairing of *Echinostoma revolutum* (Trematoda) adults. *Zeitschrift fur Parasitenkunde* **70**, 297-302.

Fried, B. & Roberts T.M. (1972). Pairing of *Leucochloridiomorpha constantiae* (Mueller, 1935) (Trematoda) *in vitro*, in the chick and on the chorioallantois. *The Journal of Parasitology* **58**, 88-91.

Fried, B. & Sousa, K.R. (1990). Single and five worm infections of *Echinostoma caproni* (Trematoda) in the ICR mouse. *International Journal for Parasitology* **20**, 125-126.

Fried, B. & Stromberg, B.E. (1971). Egg shell precursors in Trematodes. *Proceedings of the Helminthological Society of Washington* **38**, 262-264.

Fried, B., Tancer, R.B. & Fleming, S.J. (1980). *In vitro* pairing of *Echinostoma revolutum* (Trematoda) metacercariae and adults and characterization of worm products involved in chemoattraction. *The Journal of Parasitology* **66**, 1014-1018.

Fried, B. & Weaver, L.J. (1969). Exposure of chicks to the metacercariae of *Echinostoma revolutum* (Trematoda). *Proceedings of the Helminthological Society of Washington* **36**, 153-155.

Fried, B . & Wilson, B.D. (1981a). Intraspecific and interspecific pairing of *Echinostoma revolutum* (Trematoda) and *Zygocotyle lunata* (Trematoda) adults *in vitro*. *Proceedings of the Helminthological Society of Washington* **48**, 243-245.

Fried, B. & Wilson, B.D. (1981b). Pheromonal responses of *Echinostoma revolutum* in the absence of non-tactile behaviour. *Proceedings of the Helminthological Society of Washington* **48**, 251-253.

Fried, B. & Wilson, B.D. (1981). Weight of domestic chicks infected with *Echinostoma revolutum* (Trematoda) or *Zygocotyle lunata* (Trematoda). *Proceedings of the Helminthological Society of Washington* **48**, 97-98.

Fritsch, H.A.R. & Van Noorden, S. & Pearse, A.G.E. (1979). Localisation of somatostatin, substance P and calcitonin like immunoreactivity in the neural ganglion of *Ciona intestinalis* L. (Ascidiaeceae). *Cell Tissue Research* **202**, 263-274.

Fujino, T. & Ishii, Y. (1982). Ultrastructural studies on spermatogenesis in a parthenogenetic type of *Paragonimus westermani* (Kerbert, 1878) proposed as *P. pulmonalis* (Baelz 1880). *The Journal of Parasitology* **68**, 433-441.

Gaillarde, B. (1985). Techniques d'etude des schistosomes et des echinostomes. Recherches exprimentales sur le development de

Echinostoma caproni Richard, 1964. Diplome d'etudes superieures, Universite de Perpignan.

Gohar, H.A.F. & Gindy, H.I. (1961). The ecology of the Egyptian snail vectors of bilharziasis and fascioliasis. I. Physical factors. *Proceedings of the Egyptian Academy of Science* **15**, 70-78.

Grant, W.C., Harkema, R. & Muse, K.E. (1976). Ultrastructure of *Pharyngostomoides procyonis* (Harkema 1942, Oiplostomatidae). I. Observations on the male reproductive system. *The Journal of Parasitology* **62**, 39-49.

Greenberg, M.J. & Price, D.A. (1983). Invertebrate neuropeptides : Native and naturalised. *Animal Review of Physiology* **45**, 271-288.

Gremigni, V. & Domenici, L. (1974). Electron microscopical and cytochemical study of vitelline cells in the freshwater triclad *Dugesia lugubris*. I. Origin and morphogenesis of coccoon-shell globules. *Cell and Tissue Research* **150**, 261-270.

Gresson, R.A.R. (1965). Spermatogenesis in the hermaphroditic Digenea (Trematoda). *Parasitology* **55**, 117-125.

Grimmelikhijzen, C.J.P. (1984). Peptides in the nervous system of coelenterates. In : Falkner, S, Harkansen, R. and Sundler, F. eds, Evolution and tumour pathology of the neuroendocrine system Amsterdam, Elsevier.

Grimmelikhuijzen, C.J.P., Balfe, A., Emson, P.C., Powell, D. & Sundler, F. (1981). Substance P-like immunoreactivity in the nervous system of *Hydra*. *Histochemistry* **71**, 325-333.

Grimmelikhuijzen, C.J.P., Dockray, G.J. & Yanaihara, N. (1981). Bombesin like-immunoreactivity in the nervous system of *Hydra*. *Histochemistry* **73**, 171-180.

Gude, W. D., Cosgrove, G.E. & Hirsch, G.P. (1982). Histological atlas of the laboratory mouse. Plenum Press.

Gustafsson, M.KS. (1987). Immunocytochmeical demonstration of neuropeptides and serotonin in the nervous system of adult *Schistosoma manasoni*. *Parasitology Research* **74**, 169-174.

H.MSO. (1969). Fish toxicity tests. Her Majestie's Stationery Office Leaflet Number Dd. 139779 K36 12/69.

Halton, D.W. (1967). Observations on the nutrition of digenetic trematodes. *Parasitology* **57**, 639-660.

Halton, D.W., Stranock, S.D. & Hardcastle, A. (1974). Viteliine cell development in monogenean parasites. *Zeitschrift fur Parasitenkunde* **45**, 45-61.

Halvorsen, O. & Andersen, K. (1974). Some effects of population density in infections of *Diphyllobothrium dentriticum* (Nitzsch) in golden hamsters (*Mesocricetus auraus*, Waterhouse) and common gull (*Larus canus* L.). *Parasitology* **69**, 149-160.

Hanna, R.E.B. (1980). *Fasciola hepatica* : Autoradiography of protein synthesis, transport and secretion by the tegument. *Experimental Parasitology* **50**, 297-304.

Hanna, R.E.B. (1976). *Fasciola hepatica* : a light and electron microscope autoradiographic study of the incorporation of

monosaccharides into glycogen and glycoprotein. *Experimental Parasitology* **39**, 204-213.

Hanna, R.E. B. & Threadgold, L.T. (1976). *Fasciola hepatica* : stereological analysis of effects of certain metabolic inhibitors on synthesis of secretory bodies by the tegument of tissue slices. *Experimental Parasitology* **39**, 106-114.

Haque, M. & Siddiqui, A.H. (1978). Histopathology of gastrointestinal trematode parasites of pig and man. *Indian Journal of Parasitology* **2**, 97-99.

Harris, K.R. Cheng, T.C. & Cali, A. (1974). An electron microscope study of the metacercariae and adult of *Leucochloridiomorpha constantiae* (Trematoda: Brachylaemidae). *Parasitology* **68**, 57-67.

Hashiguchi, Y., Takei, T. & Miyazuki, I. (1969). Studies on a single worm infection of albino rats with *Paragonimus ohirai* Miyazaki, 1939. *Japanese Journal of Parasitology* **18**, 612-617.

Hendow, H.T. & James B.L. (1988). Ultrastructure of spermatozoon and spermatogenesis in *Maritrema linguilla* (Digenea : Microphallidae). *International Journal for Parasitology* **18**, 53-63.

Hendow, H.T. & James, B.L. (1989). Ultrastructure of vitellarium, vitellogenesis and associated ducts in *Maritrema linguilla* (Digenea : Microphallidae). *International Journal for Parasitology* **19**, 489-497.

Henley, C., Costello, D.P., Thomas, M.B. & Newton, W.D. (1969). The '9+1' pattern of microtubules in spermatozoa of *Mesotoma*

(Platyhelminths, Turbellaria). *Proceedings of the National Academy of Sciences* **64**, 849-856.

Hesselberg, C.A & Andreassen, J. (1975). Some influences of population density on *Hymenolepis diminuta* in rats. *Parasitology* **71**, 517-523

Heyneman, D., Lim, H.K. & Jeyarasasingam, U. (1972). Antagonism of *Echinostoma liei* (Trematoda : Echinostomatidae) against the trematodes *Paryphostomum segregatum* and *Schistosoma mansoni*. *Parasitology* **65**, 223-233.

Hilarrio, J.S. & Wharton, L.D. (1917). *Echinostoma ilocanum* (Garrison). A report of five cases and a contribution to the anatomy of the fluke. *Phillipine Journal of Science* **12**, 203.

Hockley, D.J. (1973). Ultrastructure of the tegument of *Schistosoma*. *Advances in Parasitology* **11**, 233-305.

Holmes, J.C. (1983). Evolutionary relationships between parasitic helminths and their hosts. In 'Coevolution' (Editors, D.J., Futuyama and M. Slatkin), Sinauer Publishers, Massachusetts, U.S.A.

Holmes, J.C. (1973). Site selection of parasitic helminths : interspecific interactions, site segregation, and their importance to the development of helminth communities. *Canadian Journal of Zoology* **51**, 333-347.

Hoole, D. & Mitchell, J.B. (1981). Ultrastructural observations on the sensory papillae of juvenile and adult *Gorgoderina vitellobia* (Trematoda : Gorgoderidae). *International Journal for Parasitology* **11**, 411-417.

Hosier, D.W. & Fried, B. (1986). Infectivity, growth and distribution of *Echinostoma revolutum* in Swiss Webster and ICR mice. *Proceedings of the Helminthological Society of Washington*, **53**, 173-176.

Hoste, H., Kerboeuf, D. & Parodi, A.L. (1988). *Trichostrongylus colubriformis* : Effects on villi and crypts along the whole small intestine in infected rabbits. *Experimental Parasitology* **67**, 39-46.

Howell, M.J. & Bearup, A.J. (1967). The life histories of two bird trematodes of the family Philophthalmidae. *Proceedings of the Linnean Society of New South Wales* **9**, 182-194.

Hsu, K.C., Lie, K.J. & Basch, P.F. (1968). The life history of *Echinostoma rodriguesi* sp. n. (Trematoda : Echinostomatidae). *The Journal of Parasitology* **54**, 333-339.

Huffman, J.E., Alcaide, A. & Fried, B. (1988). Single and concurrent infections of the golden hamster, *Mesocricetus auratus*, with *Echinostoma revolutum* and *E. liei* (Trematoda : Digenea). *The Journal of Parasitology* **74**, 604-608.

Huffman, J.E. & Fried, B. (1990). *Echinostoma* and Echinostomiasis. *Advances in Parasitology* **29**, 215-269.

Huffman, J.E., Iglesias, D. & Fried, B. (1988). *Echinosoma revolutum* : Pathology of extraintestinal infection in the golden hamster. *International Journal for Parasitology* **18**, 873-874.

Huffman, J.E., Michos, C. & Fried, B. (1986). Clinical and pathological effects of *Echinostoma revolutum* (Digenea: Echinostomatidae) in

the golden hamster, *Mesocricetus auratus*. *Parasitology* **93**, 505-515.

Hyman, L.H. (1951). The Invertebrates, vol. 2 Platyhelminths and Rhyncocoela. New York : McGraw-Hill.

Irwin, S.W. & Threadgold, L.T. (1970). Electron-microscope studies on *Fasciola hepatica* VIII. The development of the vitelline cells. *Experimental Parasitology* **28**, 399-411.

Jamieson, B.G.M. & Daddow, L.M. (1982). The ultrastructure of the spermatozoan of *Neochasmus* sp. (Cryptogonimidae, Digenea, Trematoda) and its phylogenetic significance. *International Journal for Parasitology* **12**, 547-559.

Jarrett, E.E.E., Jarrett., W.F.H. & Urquahat, G.M. (1968). Quantitative studies on the kinetics of establishment and expulsion of intestinal nematode populations in susceptable and immune hosts. *Nippostronglus brasiliensis* in the rat. *Parasitology* **58**, 625-639.

Jennings, J.B. (1968). 'Nutrition and digestion'. in Chemical Zoology, II. *Rotifera Colenterata* and Platyhelminths editors M. Florkin and B.T. Scheer, Academic Press: New York.

Jeyarasasingam, U., Heyneman, D., Lim, H.K. & Mansour, N. (1972). Life cycle of a new echinostome from Egypt *Echinostoma liei* (Trematoda: Echinostomatidae). *Parasitology* **65**, 203-222.

Johri, L.N. & Smyth, J.D. (1956). A histochemical approach to the study of helminth morphology. *Parasitology* **46**, 107-117.

Jones, A.W., Fitzgerald, M.D., Proffitt, M.R., Tan, B.D. & Ward, H.L. (1971). Prolonged selfing of *Hymenolepis microstoma* (Cestoda). *Experimental Parasitology* **29**, 223-229.

Jourdane, J. & Kulo, S.D. (1981). Etude experimentale du cycle biologique de *Echinostoma togoensis* n. sp., parasite a l' etat larvaire de *Biomphalaria pfeifferi* au Togo. *Annales de Parasitologie Humaine et Comparee* **56**, 477-488.

Jourdane, J. & Kulo, S.D. (1982). Perspectives d'utilisation de *Echinostoma togoensis* Jourdane et Kulo, 1981 dans le controle biologique de la bilharziose intestinale en Afrique. *Annales de Parasitologie Humaine et Comparee* **57**, 443-451.

Jourdane, J., Mounkassa, J.B. & Imbert-Establet, D. (1990). Influence of intramolluscan larval stages of *Echinostoma liei* on the infectivity of *Schistosoma mansoni* cercariae. *Journal of Helminthology* **64**, 71-74.

Kanev, I. (1985). On the morphology, biology, ecology and taxonomy of the *E. revolutum* group (Trematoda: Echinostomatidae; *Echinostoma*). D.Sc. Thesis, Bulgarian Academy of Sciences, Sofia Bulgaria.

Karlsson, C., Davies, H., Ohman, J. & Anderssen, U. (1973). L.K.B. Application note **75**, 1-13.

Kennedy, C.R. (1976). 'Reproduction and dispersal', in Ecological aspects of Parasitology ed. C.R. Kennedy North Holland Publishing Company, Amsterdam.

Kessel, R.G. (1966). The association between microtubules and nuclei during spermiogenesis in the dragonfly. *Journal of Ultrastructure Research* **16**, 293-304.

Keymer, A. E. (1982). Density-dependent mechanisms in the regulation of intestinal helminth populations. *Parasitology* **84**, 573-587.

Keymer, A.E. & Slater, A.F.G. (1987). Helminth fecundity : density dependence or statistacal illusion? *Parasitology Today* **3**, 56-58.

Kim, S. & Fried, B. (1989). Pathological effects of *Echinostoma caproni* (Trematoda) in the domestic chick. *Journal of Helminthology* **63**, 227-330.

Kino, H. (1984). Parasite density and the fecundity of *Angiostrongylus cantonensis* in rats. *Parasitology* **89**, 275-285

Kinsella, J.M. (1971). Growth, development and intraspecific variation of *Quinqueserialis quinquiserialis* (Trematoda : Notocotylidae) in rodent hosts. *The Journal of Parasitology* **57**, 62-70.

Kishore, N. & Sinha, D.P. (1982). Observations on *Echinostoma revolutum* infection in the rectum of domestic ducks (*Anas platyrhynchos domesticus*). *Agricultural Science Digest* **2**, 57-60.

Koie, M. (1987). Scanning electron microscopy of rediae, cercariae, metcacercariae and adults of *Mesorchis denticulatus* (Rudolphi, 1802) (Trematoda, Echinostomatidae). *Parasitology Research* **73**, 50-56.

Kuris, A.M. (1973). Biological control: Implications of the analogy between the trophic interactions of insects pest-parasitoids and snail trematode systems. *Experimental Parasitology* **33**, 365-379

Kuris, A.M. (1980a). *Echinostoma liei* miracidia and *Biomphalaria glabrata* snails: Effect of egg age, habitat heterogenity, water quality and volume on infectivity. *International Journal for Parasitology* **10**, 21-25

Kuris. A.M. (1980b). Effect of exposure to *Echinostoma liei* miracidia on growth and survival of young *Biomphalaria* snails. *International Journal for Parasitology* **10**, 303-308.

Kuris, A.M. & Warren, J. (1980). *Echinostoma* cercarial penetration and metacercarial encystment as mortality factors for a second intermediate host, *Biomphalaria glabrata*. *The Journal of Parasitology* **66**, 630-635.

Larsson, L.I., Fahrenkruy, J., Schaffalitzky de Mucadell, O., Sundler, F., Hakanson R., & Rehfeld, J.F. (1976). Localisation of vasoactive intestinal polypeptide (VIP) to central and peripheral neurons. *Proceedings of the National Academy of Science* **73**, 3197-3200.

Lie, K.J. (1967). Antagonism of *Paryphostomum segregatum* rediae to *Schistosoma mansoni* sporocyst in the snail *Biomphalaria glabrata*. *The Journal of Parasitology* **53**, 969-976.

Lie, K.J. (1965). Studies on Echinostomatidae (Trematoda) in Malaya. IX The Mehlis' gland complex in echinostomes. *The Journal of Parasitology* **51**, 789-792.

Lie, K.J. & Basch, P.F. (1967). The life history of *Echinostoma paraensei* sp. n. (Trematoda : Echinostomatidae). *The Journal of Parasitology* **53**, 1192-1199.

Lie, K.J., Basch, P.F., Heyneman, D., Beck, A.J. & Audy, J.R. (1968). Implications for trematode control of interspecific larval antagonism within snail hosts. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **62**, 299-319.

Lie, K.J., Basch, P.F. & Hoffman, M.A. (1967). Antagonism between *Paryphostomum segregatum* and *Echinostoma barbosai* in the snail *Biomphalaria straminea*. *The Journal of Parasitology* **53**, 1205-1209.

Lie, K.J., Kuro, E.B. & Owyang, C.K. (1970). A field trial to test the possible control of *Schistosoma spindale* by means of interspecific trematode antagonism. *South East Asian Journal of Tropical Medicine and Public Health* **2**, 237-243.

Lie, K.J. Kuro, E.H. & Owyang, C.K. (1971). Further trial to control *Schistosoma spindale* by trematode antagonism. *Southeast Asian Journal of Tropical Medicine and Public Health* **2**, 237-243.

Lie, K.J. & Owyang, C.K. (1962). Studies on Echinostomatidae (Trematoda) in Malaya. V. Some results on studies of *Echinostoma malayanum* Leiper, 1911. UNESCO Registered Symposium of Tropical Parasitology.

Lie, K.J., Schneider, C.R., Sornmani, S., Lanza, G.R. & Impand, P. (1974a). Biological control by Trematode antagonism. I. A successful field trial to control *Schistosoma spindale* in Northeast

Thailand. *Southeast Asian Journal of Tropical Medicine and Public Health* **5**, 46-59.

Lie, K.J., Schneider, C.R., Sornmani, S., Lanza, G.R. & Impand, P. (1974b). Biological control by trematode antagonism. II. Failure to control *Schistosoma spindale* in a field trial in Northeast Thailand. *Southeast Asian Journal of Tropical Medicine and Public Health* **5**, 60-64.

Lie, K.J. & Virik, H.K. (1963). Human infection with *Echinostoma malayanum* Leiper, 1911 (Trematoda: Echinostomatidae). *Journal of Tropical Medicine and Hygiene* **66**, 77-82.

Lim, K.J. & Heyneman, D. (1972). Intramolluscan intertrematode antagonism : a review of factors influencing the host parasite system and its possible role in biological control. *Advances in Parasitology* **10**, 192-268.

Lloyd, S & Soulsby, E.J. L. (1987). 'Immunobiology of gastrointestinal nematodes of ruminants' In Immune Responses in Parasitic Infections: Immunology, Immunopathology,and Immunoprophylaxis, vol. 1: Nematodes. (Ed. E.J.L. Soulsby).

Mabus, J., Huffman, J.E. & Fried, B. (1988). Humoral and cellular response to infection with *Echinostoma revolutum* in the golden hamster, *Mesocricetus auratus*. *Journal of Helminthology* **62**, 127-132.

Macdonald, G. (1965). The dynamics of the helminth infections with special reference to schistosomes. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **59**, 489-506.

MacFarlane, W.V. (1939). Life cycle of *Coitcaecum anaspidus* Hickman, a New Zealand digenetic trematode. *Parasitology* 31, 172-184.

Mackiewicz, J.S. (1981). Caryophyllidea (Cestoda) : Evolution and classification. *Advances in Parasitology* **19**, 139-206.

Magee, R.M., Faiweather, I., Johnston, C.F., Halton, D.W. & Shaw, C. (1989). Immunocytochemical demonstration of neuropeptides in the nervouss system of the liver fluke *Fasciola hepatica* (Trematoda, Digenea). *Parasitology* **98**, 227-238.

Mahendrasingam, S. Fairweather, I. & Halton, D.W. (1989). Vitelline cell development in *Trilocularis acanthiaevulgaris* (Cestoda : Tetraphyllidea). Abstrascts of the British Society for Parasitology Spring Meeting, Southampton University p. 37.

Maule, A.G., Halton, D.W., Johnston, C.F., Fairweather, I. & Shaw, C. (1989). Immunocytochemical demonstration of neuropeptides in the fish-gill parasite, *Diclidophora merlangi* (Monogenoidea). *International Journal for Parasitology* **19**, 307-316.

McCarthy, A.M. (1989). The biology and transmission dynamics of *Echinoparyphium recurvatum* (Digenea: Echinostomatidae). Phd Thesis, University of London (King's College).

Mcdowall, A.A. & James, B.L. (1988). The functional morphology of the circumoral spines of *Timoniella imbutiforme* (Molin, 1895) Brooks 1980 (Digenea : Acanthostomidae). *International Journal for Parasitology* **18**, 523-531.

Mclaren, D.J. (1973). The structure and development of the spermatozoon of *Dipetalonema viteae* (Nematoda : Filarioidea). *Parasitology* **66**, 447-463.

Mettrick, D. F. (1971). Effect of host dietry constituents on intestinal pH and the migrational behaiviour of the rat tapeworm *Hymenolepis diminuta*. *Canadian Journal of Zoology* **49**, 1513-1525.

Michael, E. & Bundy, D.A.P. (1989). Density dependence in establishment, growth and worm fecundity in intestinal helminthiasis : the population biology of *Trichuris muris* (Nematoda) infection in CBA/ Ca mice. *Parasitology* **98**, 451-458.

Michaels, R.M. (1969). Mating of *Schistosoma mansoni in vitro*. *Experimental Parasitology* **25**, 58-71.

Miyazaki, I. (1978). Two types of lung fluke which have been called *Paragonimus westermani* (Kerbert, 1878). *Medical Bulletin of Fakuoka University* **5**, 251-263.

Miyazaki, I. Terasaki, K. & Habe, S. (1981). Comparison of single-worm infection to cats between *Paragonimus westermani* and *P. pulmonalis* . *Medical Bulletin of Fukuoka University* **8**, 405-416.

Mohandas, A. & Nadakal, A.M. (1978). *In vivo* development of *Echinostoma malayanum* Leiper, 1911 with notes on effects of population density, chemical composition and pathogenicity and *in vitro* excystment of the metacercariae (Trematoda: Echinostomatidae). *Zeitschift fur Paraitenkunde* **55**, 139-151.

Moravec, F., Barus, V., Rysavy, B. & Yousif, F. (1974). Observations on the development of two echinostomes, *Echinoparyphyium recurvatum* and *Echinostoma revolutum*, the antagonists of human schistosomes in Egypt. *Folia Parasitologica* **21**, 107-126.

Morris, G.P. & Threadgold, L.T. (1967). A presumed sensory structure associated with the tegument of *Schistosoma mansoni*. *The Journal of Parasitology* **53**, 537-539.

Moseley, C. & Nollen, P.M. (1973). Autoradiographis studies on the reproductive system of *Philophthalmus hegeneri* Penner and Fried, 1963. *The Journal of Parasitology* **59**, 650-654.

Moussa, A.H. & El-Hassan, A.A.A. (1972). The effect of water temperature on the snail intermediate hosts of schistosomiasis in Egypt. *Journal of the Egyptian Medical Association* **55**, 148-165.

Mutafova, T. & Kanev, I. (1983). Karyotypes of an echinostome with features of *Echinostoma barbosai* Lie and Basch, 1966 (Trematoda : Echinostomatidae) found in Bulgaria. *Khelmintobgiya* **16**, 42-45.

Muttafova, T. & Kanev, I. (1986). On the *Echinostoma revolutum* (Froelich, 1802) and *Echinostoma echinatum* (Zeder, 1803), (Trematoda : Echinostomaidae) karyotypes. *Helminthology, Bulgarian Academy of Sciences, Sofia* **22**, 41.

Mutt, V. & Said, S.I. (1974). Structure of the porcine vasoactive intestinal octacosapeptide. *European Journal of Biochemistry* **42**, 581-589.

Nakane, P.K. & Pierce, G.B. (1967). Enzyme labelled antibodies : Preparation and application for the localization of antigens. *Journal of Histochemistry and Cytochemistry* **14**, 929-931.

Nassi, H. (1978). Donneses sur le cycle biologique de *Ribeiroia marini guadaloupensis* n.ssp., trematode sterilisant *Biomphalaria glabrata* en Guadeloupe. Entretien du cycle en vue dun controle eventuael des populations de mollusques. *Acta Tropica* **35**, 41-56

Nassi, H., Pointer, J.P. & Golvan, Y.J. (1979). Bilan d'un essai de controle de *Biomphalaria glabrata* en Guadeloupe a l'aide d'un Trematode sterilisant. *Annales Parasitoogy* **54**, 185-192.

Nelson, P.D. & Fried, B. (1979). Growth and reproduction in single and ten worm infections of *Zygocotyle lunata* (Trematoda) in the chick. *Zeitschrift fur Parasitenkunde* **60**, 97-100.

Nollen, P.M. (1971a). Studies on growth and infection of *Philophthalmus megalurus* (Cort, 1914) (Trematoda) in chicks. *The Journal of Parasitology* **57**, 261-266.

Nollen, P.M. (1968). Autoradiographic studies on reproduction in *Philophthalmus megalurus* (Cort, 1914) (Trematoda). *The Journal of Parasitology* **54**, 43-48.

Nollen, P.M. (1971b). Viability of a self fertilising strain of *Philophthalmus megalurus* (Trematoda: Digenea). *The Journal of Parasitology* **57**, 1222-1226.

Nollen, P.M. (1975). Studies on the reproductive system of *Hymenolepis diminuta* using autoradiography and transplantation. *The Journal of Parasitology* **61**, 100-104.

Nollen, P.M. (1983). Patterns of sexual reproduction among parasitic platyhelminths. *Symposia of the British Society for Parasitology* **20**, 99-120.

Nollen, P.M. & Alberico, R.A. (1972). Absence of testes in *Gorgoderina attenuata*. *The Journal of Parasitology* **58**, 1011-1012.

Nollen, P.M. & Pyne, J.L. (1979). Observations on spermatogenesis and inseminative behaviour of *Megalodicus temperatus* in frogs. *The Journal of Parasitology* **65**, 35-37.

O'Dorisio, T.M. (1978). Pathophysiology of VIP II. In 'Gut hormones' S.R. Bloom ed. Livingstone, Edinburgh.

Odaibo, A.B., Christensen, N.O. & Ukoli, M.A. (1988). Establishment, survival and fecundity in *Echinostoma caproni* (Trematoda) infections in NMRI mice. *Proceedings of the Helminthological Society of Washington* **55**, 265-269.

Ogbe, M.G. (1987). Scanning electron microscopy of tegumental surfaces of adult and developing *Schistosoma margrebowiei* Le Roux, 1933. *International Journal for Parasitology* **12**, 191-198.

Ohman, C. (1966). The structure and function of the adhesive organ in strigeid trematodes. IV *Holostephasus luhei* Szidat, 1936. *Parasitology* **56**, 481-491.

Orido, Y. (1988). Fertilisation and oogenesis of lung fluke *Paragonimus ohirai* (Trematoda : Troglotrematidae). *International Journal for Parasitology* **18**, 973-980.

Orido, Y. & Hata, H. (1988). Cytological and cytofluorometrical studies on gametogenesis of *Paragonimus ohirai* (Trematoda : Troglotrematidae). *International Journal for Parasitology* **18**, 98-101.

Osbourne, N.N., Patel, S. & Dockray, G.J. (1982). Immunohistochemical demonstration of peptides serotonin and depomone-Beta-hydroylase-like material in the nervous system of the leech *Hirudo medicinalis*. *Histochemistry* **75**, 573-583.

Page, M.R., Nadakavukaren, M.J. & Huizinga, H.W. (1980). *Ribeiroia marini* : surface ultrastructure of rediae, cercaria and adult. *International Journal for Parasitology* **10**, 5-13.

Phillips, D.M. (1974). Spermiogenesis. Academic Press.

Polak, J.M. Pearse, A.G.E., Geruad, J.C. & Bloom, S.R. (1974). Cellular localisation of vasoactive peptide in mamalian and avian gastrointestinal tract. *Gut* **15**, 720-724.

Price, D.A. & Greenberg, M.J. (1980). The pharmacology of the molluscan cardioexcitatory neuropeptide FMRFamide. *General Pharmacology* **11**, 237-241.

Price, P.W. (1980). Evolutionary biology of parasites. Princeton: Princeton University Press.

Radlett, A.J. (1979). Excystation of *Notocotylus attenuatus* (Ruldolphi, 1809) Kossack, 1911 (Trematoda : Notocotylidae) and their localisation in the caecum of the domestic fowl. *Parasitology* **79**, 411-416.

Rao, K.H. (1963). The structure of the Mehlis' gland complex and histochemistry of *Artyfechinostomum mehrai* (Faruqui) (Trematoda: Echinostomatidae) from a girl at Andhra Pradesh, India. *Parasitology* **53**, 1-5.

Read, C. P. (1971). 'The microcosm of intestinal helminths' in Ecology and Physiology of Parasites, editor A.M. Fallis, Adam Hilger Ltd. p.188-200.

Rees, F.G. (1979). The ultrastructure of the spermatozoon and spermiogenesis in *Cryptocotyle lingua* (Digenea : Heterophyidae). *International Journal for Parasitology* **9**, 405-419.

Reuter, M., Karhi, T. & Sehot, L.P.C. (1984). Immunocytochemical demonstration of peptidergic neurones in the central and periphery nervous systems of the flatworm *Microstomum lineare* with antiserum to FMRF-amide. *Cell and Tissue Research* **238**, 431-436.

Rhode, K. (1979). A critical evaluation of intrinsic and extrinsic factors responsible for niche restriction in parasites. *American Naturalist* **114**, 648-671.

Rhode, K. (1966). Sense receptors of *Multicotyle purvisi* Dawes 1941 (Trematoda : Aspidobothria). *Nature* **211**, 820-822.

Rhode, K. (1968). The nervous system of *Multicotyle purvisi* Dawes 1941 (Aspidogastrea) and *Diaschistorchis mutitesticularis* Rohde 1962 (Digenea). *Zeitschrift fur Parasitenkunde* **30**, 78-94.

Richard, J. (1964). Trematodes d'oiseaux de Madagascar (note III). Especes de la famille Echinostomatidae Poche, 1926. *Annales de Parasitologie Humaine et Comparee* **34**, 607-620.

Richard, J. & Brygoo, E.R. (1978). Life cycle of the trematode *Echinostoma caproni* Richard 1964. *Annales de Parasitologie Humaine et Comparee* **53**, 265-278.

Richard, J., Klein, M.J. & Stoeckel, M.E. (1989). Neural and glandular localisation of substance P in *Echinostoma caproni* (Trematoda: Digenea). *Parasitology Research* **75**, 641-648.

Richard, J. & Voltz, A. (1987). Preliminary data on the chromosomes of *Echinostoma caproni* Richard, 1964 (Trematoda : Echinostomatidae). *Systematic Parasitology* **19**, 169-172.

Riddell, J.H., Whitfield, P.J., Balogun, M.A. & Thorndyke, M.C. (1991). FMRFamide-like peptides in the nervous and endocrine systems of the digenean helminth *Echinostoma liei*. *Acta Zoologica* **72**, 1-5.

Rim, H.J. (1982). CRC Handbook Series in Zoonoses (Ed. G.V. Hillyer & C.E. Hopla) CRC Press Florida U.S.A.

Roberts, L.S. (1961). The influence of population density patterns and physiology of growth in *Hymenolepis diminuta* (Cestoda : Cyclophyllidea) in the definitive host. *Experimental Parasitology* **11**, 332-371.

Roberts, L.S. & Insler, G.D. (1982). Developmental physiology of cestodes XVII. Some biological properties of putative crowding factors in *Hymenolepis diminuta*. *The Journal of Parasitology* **68**, 263-270.

Roberts-Thompson, I.C. & Mitchell G.F. (1978). Giardiasis in mice I. Prolonged infections in certain mouse strains and hypothymic (nude) mice. *Gastroenterology* **75**, 42-46

Robinson, R.D. & Halton, D.W. (1982). Fine structural observations on spermatogenesis in *Corrigia vitta* (Trematoda : Dicrocoelidae). *Zeitschrift fur Parasitenkunde* **68**, 53-72.

Rogers, W.A. & Ulmer, M.J. (1962). Effects of continued selfing in *Hymenolepis nana* (Cestoda). *Proceedings of the Iowa Academy of Sciences* **69**, 557-571

Ross, G.C. (1976). Isoenzymes in *Schistosoma* spp : LDH, MDH and acid phosphatases separated by isoelectric focussing in polyacrylamide gel. *Comparative Biochemistry and Physiology* **55B**, 343-346.

Ross, G.C., Fried, B. & Southgate, V.R. (1989). *Echinostoma revolutum* and *E. liei* : observations on enzyme pigments. *Journal of Natural History* **23**, 977-981.

Ross, J.G. (1965). Experimental infections of cattle with *Fasciola hepatica* : A comparison of low and high infection rates. *Nature* **208**, 907.

Rysavy, B., Ergens, J., Groschaft, J., Moravec, F., Yousif, F. & El-Hassan, A.A. (1973). Preliminary report on the possibilities of utilizing competition of larval schistosomes and other larval trematodes in the intermediate hosts for the biological control of schistosomiasis. *Folia Parasitologica* **20**, 293-296.

Said, S.I. (1981). VIP overview. In 'Gut hormones' S.R. Bloom and J.M. Polak editors, Livingstone Edinburgh.

Said, S.I. & Mutt, V. (1970). Potent peripheral and splanchnic vasodilator peptide from normal gut. *Nature* **225**, 863-864.

Saito, S. (1984). Development of *Echinostoma hortense* in rats with special reference to genital organs. *Japanese Journal of Parasitology* **33**, 191-201.

Sakaguchi, Y. (1980). Karyotype and gametogenesis of the common liver fluke. *Fasciola* sp. *Japanese Journal of Parasitology* **29**, 507-513.

Sasada, K. (1978). Studies on the chromosomes of parasitic helminhts (II). Triploidy and cytological mechanism of parthenogenesis in *Diphyllobothrium erinacei* (Cestoda : Diphyllobothridae). *Japanese Journal of Parasitology* **27**, 547-560.

Schelfer, W.C. (1979). Statistics for the biological sciences. Second edition, Addison-Weeley Publishing Company Incorporated.

Schilt, J., Richoux, J.P. & Dubois, M.P. (1981). Demonstration of peptides immunologically related to vertebrate neurohormones in *Dugesia lugbris* (Turbellaria, Tricladida). *General and Comparative Endocrinology* **43**, 331-335.

Scott, M.E. & Lewis J.W. (1987). Population dynamics of helminth parasites in wild and laboratory rodents. *Mammal Review* **17**, 95-103.

Senger, C.M. (1954). Notes on the growth, development and survival of two echinostome trematodes. *Experimental Parasitology* **3**, 491-496.

Seo, B.S., Hong, S.T., Chai, J.Y. & Lee, S.Y. (1983). Studies on intestinal trematodes in Korea. *The Korean Journal of Parasitology* **21**, 219-223.

Shapiro, J.E., Hershenov, B.R. & Tulloch, G.S. (1961). The fine structure of *Haematoloechus* spermatozoan tail. *Journal of Biophysical and Biochemical Cytology* **9**, 211-217.

Shaw, M.K. (1987). *Schistosoma mansoni* : Vitelline gland development in females from single sex infections. *Journal of Helminthology* 61, 253-259.

Shiner, M. & Doniach, I. (1960). Histopathological studies in steatorrhea. *Gastroenterology* **38**, 419-440

Short, R.B., (1952). Uniparental miracidia of *Schistosomatium douthitti* and their progeny (Trematoda: Schistosomatidae). *American Midland Naturalist* **48**, 55-68.

Short, R.B. & Menzel, M.Y. (1959). Chromosomes in parthenogenetic miracidia and embryonic cercariae of *Schistosomatium douthitti*. *Experimental Parasitology* **8**, 249-264.

Silveira, M. & Porter, K.R. (1964). The spermatozoids of flatworms and their microtubular systems. *Protoplasma* **59**, 240-265.

Simonsen, P.E. & Andersen, B.J. (1986). *Echinostoma revolutum* : dynamics of the antibody attack to the surface of an intestinal trematode. *International Journal for Parasitology* **16**, 475-482.

Simonsen, P.E., Christensen, N.O. & Koie, M. (1989). *Echinostoma caproni* in mice : studies on the attachment site of an intestinal trematode. *International Journal for Parasitology* **19**, 561-566.

Smales, L.R. & Blankespoor, H.D. (1984). *Echinostoma revolutum* (Froelich, 1802) Looss, 1899 and *Isthmiophora melis* (Schrank,

1788) Luhe, 1909 (Echinostomatidae, Digenea) : Scanning electron microscopy of the tegumental surfaces. *Journal of Helminthology* **58**, 187-195.

Smyth, J.D. (1962). Introduction to animal parasitology. C. C. Thomas, Springfield III.

Smyth, J.D. (1954). Studies on tapeworm physiology. VII. Fertilisation of *Schistocephalus solidus in vitro*. *Experimental Parasitology* **3**, 64-71.

Smyth, J.D. & Clegg, J.A. (1959). Egg shell formation in trematodes and cestodes. *Experimental Parasitology* **8**, 286-323.

Smyth, J.D. & Halton, D.W. (1986). The physiology of trematodes. Second Edition. Cambridge University Press Cambridge and New York.

Smyth, J.D. & Smyth, M.M. (1969). Self-insemination in *Echinococcus granulosus in vivo*. *Journal of Helminthology* **43**, 383-388.

Sogandares-Bernal, F. (1966). Studies on American paragonimiasis IV. Observations on the pairing of adult worms in laboratory infections of cats. *The Journal of Parasitology* **52**, 701-703.

Southgate, V. R., Ross, G.C. & Knowles, R.J. (1981). On *Schistosoma leiperi* Le Roux, 1955: Scanning electron microscopy of adult worms, compatability with species of *Bulinus*, development in *Mesocricetus auratus* and isoenzymes. *Zeitshrift fur Parasitenkunde* **66**, 63-81.

Spry, C.J.F. (1988). Eosinophils. A comprehensive review and guide to the scientific and medical literature. Oxford University Press.

Sternberger, L.A. (1974). Immunocytochemistry. In 'Foundations of immunology' series (ed. A. Oster and L. Weiss) Englewood, Cliffs New Jersey. Prentice Hall.

Stone, J.V., Mordue, W., Broomfield, C.E. & Hardy, P.M. (1978). Structure activity relationship for the lipid mobilising action of the locust adipokinetic hormone. *European Journal of Biochemistry* **89**, 195-202.

Sukhdeo, M.V.K., Sangster, N.C. & Mettrick, D.F. (1988). Permanent feeding sites of adult *Fasciola hepatica* in rabbits? *International Journal for Parasitology* **18**, 509-512.

Sundler, F., Hakanson, R., Alumets, J. & Walles, B. (1977). Neuronal localization of pancreatic polypeptide (PP) and vasoactive intestinal peptide (VIP) in the earthworm (*Lumbricus terrestus*). *Brain Research Bulletin* **2**, 61-65.

Symons, L.E.A. (1957). Pathology of infestation of the rat with *Nippostrongylus muris* (Yokogawa). I. Changes in the water content, dry weight and tissues of the small intestine. *Australian Journal of Biological Sciences* **10**, 374-383.

Symons, L.E.A. & Fairbain, D. (1963). Biochemical pathology of the rat jejunum parasitized by the nematode *Nippstrongylus brasiliensis*. *Experimental Parasitology* **13**, 284-304.

Tager, H.S., Markene, J., Kramer, K.J.Spiers, R.D. & Childs, C.N. (1976). Glucagon-like and insulin lile hormones of the insect neurosecrtoey system. *Biochemical Journal* **156**, 515-520

Taylor, M.G., Amin, M.B.A. & Nelson, G.S. (1969). Parthenogenesis in *Schistosoma mattheei*. *Journal of Helminthology* **43**, 197-206.

Terasaki, K., Mariyano, N., Tanis, S. & Ishida, K. (1982). Comparative studies on the karyotypes of *Echinostoma cinetorchis* and *E. hortense* (Echinostomatidae : Trematoda). *Japanese Journal of Parasitology* **31**, 569-574.

Tesana, S., Pipatphong, K., Maleewong, W. & Kaewkes, S. (1987). Scanning electron microscopy of adult *Echinostoma malayanum*. *Southeast Asian Journal of Tropical Medicine and Public Health* **18**, 233-240.

Thomas, M.B. & Henley, C. (1971). Substructure of the cortical singlet microtubules in spermatozoa of *Macrostomum* (Platyhelminths, Turbellaria) as revealed by negative staining. *The Biological Bulletin* **141**, 592-601.

Thorndyke, M.C. (1986). Immunocytochemistry and evolutioanary studies with particular reference to peptides. In 'Immunocytochemistry, modern methods and applications' (J.M. Polak and S. Van Noorden, Eds), Wright, Bristol.

Thorndyke, M.C. and Whitfield, P.J. (1987). Vasoactive intestinal polypeptide-like immunoreactive tegumental cells in the digenean helminth *Echinostoma liei* : Possible role in host-parasite interactions. *General and Comparative Endocrinology* **68**, 202-207.

Thorpe, E. (1968). Comparative enzyme histochemistry of immature stages of *Fasciola hepatica*. *Experimental Parasitology* **22**, 150-159.

Threadgold, L.T. (1967). Electron microscope studies of *Fasciola hepatica*. III. Further observations on the tegument and associated structures. *Parasitology* **57**, 633-637.

Threadgold, L.T. (1967). The tegument and associated structures of *Fasciola hepatica* L. *Quarterly Journal of Microscopical Science* **104**, 505-512.

Threadgold, L.T. (1968). The tegument and associated structures of *Haplometra cylindracea*. *Parasitology* **56**, 1-7.

Threadgold, L.T. (1976). *Fasciola hepatica* : ultrastructure and histochemistry of the glycocalyx of the tegument. *Experimental Parasitology* **39**, 119-134.

Threadgold, L.T. (1982). *Fasciola hepatica* : Stereological analysis of vitelline cell development. *Experimental Parasitology* **54**, 352-365.

Thulin, J. (1980). Scanning electron microscope observations of *Aporocotyle simplex* Odhner, 1900 (Digenea:Sanguinicolidae). *Zeitschrift fur Parasitenkunde* **63**, 27-32.

Tinsley, R.C. & Owen, R.W. (1975). Studies on the biology of *Protopolystoma xenopodis* (Monogenoidea) : the oncomiracidium and life cycle. *Parasitology* **71**, 445-463.

Tulloch, G.S. & Shapiro, J.E. (1957). The ultrastructure of the vitelline cells of *Haematoloechus*. *The Journal of Parasitology* **43**, 628-632.

Turton, J.A. (1971). Distribution and growth of *Hymenolepis diminuta* in the rat, hamster and mouse. *Zeitshrift fur Parasitenkunde* **37**, 315-329.

U.N.D.P. / World Bank / W.H.O. (1984). Special programme for research and training in tropical diseases. Report of an informal consultation on research on the biological control of snail intermediate hosts.

Ulmer, M.J. (1970). 'Site finding behaviour in helminths in intermediate and definitive hosts' in Ecology and Physiology of Parasites, editor A.M. Fallis, Adam Hilger Ltd. p.121-160.

Vasilev, I., Mikov, L., Kanev, I. & Fried, B. (1984). Comparative electrophoretic study of adult *Echinostoma revolutum* (Froelich, 1802) from Europe and the U.S.A. *Khelmintologiya* **17**, 10-15.

Voltz, A., Richard, J. & Pesson, B. (1987). A genetic comparison between natural and laboratory strains of *Echinostoma* (Trematoda) by isoenzymatic analysis. *Parasitology* **95**, 471-477.

Voltz, A., Richard, J., Pesson, B. & Jourdane, J. (1988). Isoenzyme analysis of *Echinostoma liei* : Comparison and other hybrdization with other African species. *Experimental Parasitology* **66**, 13-17.

Wakelin, D. & Wilson, M.M. (1980). Immunity to *Trichinella spiralis* in irradiated mice. *International Journal for Parasitology* **10**, 37-41.

Weather, P.R. & Wilson, R.A. (1976). The tegument of *Schistosoma mansoni* : a histochemical investigation. *Parasitology* **72**, 99-109.

Whitfield, P.J. (1979). The biology of parasitism : an introduction to the study of associating organisms. Edward Arnold (Publishers), London.

Whitfield, P.J. & Evans, N.A. (1983). Parthenogenesis and asexual multiplication among parasitic platyhelminths. *Symposia of the British Society for Parasitology* **20**, 121-160.

Williams, H.H. & McVicar, A. (1968). Sperm transfer in tetraphyllidea (Platyhelminthes : Cestoda). *Nytt Magasin for Zoologi* **16**, 61-71.

Wisnewski, N., Fried, B. & Halton, D.W. (1986). Growth and feeding of *Echinostoma revolutum* on the chick chorioallantois and in the domestic chick. *The Journal of Parasitology* **72**, 684-689.

Wright, C.A., Southgate, V.R. & Ross, G.C. (1979). Enzymes in *Schistosoma intercalatum* and the relative status of the lower Guinea and Zaire strains of the parasite. *International Journal for Parasitology* **9**, 523-528.

Yamashita, J. (1964). Echinostome. In Progress of Medical Parasitology in Japan vol. 1 Meguro Parasitological Museum, Tokyo, 289.

Yokogawa, H. Yoshimura, C. & Oshima, T. (1960). Studies on experimental infection of dogs with three metacercariae of *Paragonimus westermani*. *Japanese Journal of Parasitology* **10**, 6-13.

Yokogawa, S. Cort, W.W. & Kokogawa, M. (1960). *Paragonimus* and paragonimiasis. *Experimental Parasitology* **10**, 31-205.

Appendix 1

Aceto-orcein solution

Add 1-2g orcein (Gurr's) to 45ml hot acetic acid. When solution cool add 55ml of distilled water.

Berland's solution

19 parts glacial acetic acid

1 part 40% formalin

Borax carmine

Carmine 3 g

Borax 4 g

Distilled water 100 ml

Boil mixture to dissolve carmine then add

70% alcohol 100 ml

Filter final solution

Staining procedure

1. Fix specimens in 70% (24 hours minimum)
2. Stain in Borax carmine 10-15 minutes
3. Acid alcohol 10-15minutes
4. 70% alcohol 10-15 minutes
5. 90% alcohol 10-15 minutes
6. 100% alcohol (2 changes) 10-15 minutes each
7. Xylene 10-15 minutes

8. Xylene + Ralmount mixture 24-hours

9. Mount specimens in pure ralmount

Buffered distilled water

Add 2ml 0.2% Bromo-thymol blue to 4 litres of distilled water. Adjust to pH 7.2 adding saturated lithium carbonate solution.

Carnoy's fixative

Acetic acid	20ml
Absolute ethanol	60ml

Fast Red Salt B

Fix specimens in 70% ethanol

Take specimens through decreasing alcohol series to distilled water then stain in 1% Fast Red Salt B.

Formyl alcohol-acetic (FAA)

Acetic acid	5ml
Formalin (40%)	5ml
Ethanol (70%)	90ml

Giemsa stain

Giemsa	1g
Glycerine	66ml
Methanol	66ml

Rotate Giemsa mixture for 3 hours with glass beads in stoppered flask.

Haematoxylin and eosin

Staining procedure

1.Specimens fixed in FAA 24 hours minimum

2.Histoclear (2 changes) 5 minutes each

3.Absolute alcohol 1 minute

4.Celloidin 3-5seconds

5.Ethanol (70%) 5 minutes

6.Acid alcohol 2-3 seconds

7.Distilled water 5 minutes

8.Eosin 4 minutes

9.Absolute alcohol 1 minute

10.Butyl alcohol (2 changes) 2 minutes each

11.Ether-alcohol 5 minutes

12. Histoclear (2 changes) 2 minutes each

Mount specimens in Ralmount

Spurr resin

Vinylcyclohexane 10ml

Diglycidyl ether 6ml

Nonenylsuccinic anhydride 26ml

Dimethylaminoethanol 0.4ml

Appendix 2

Bouin's fixative

Picric acid, saturated aqueous 75ml

Formalin (40%) 25ml

Glacial acetic acid 5ml

Phosphate buffered saline

Sodium dihydrogen orthophosphate 0.156g

Di-sodium hydrogen orthophosphate 1.28g

Sodium chloride 8.5g

Distilled water 1000ml

Tris-HCl buffer (0.05M)

<u>pH 7.2 buffer</u>

0.2MTris-(hydroxymethyl)-aminomethane

25 ml

0.2 M HCl (Hydrochloric acid) 22.1 ml

Make up to 100 ml with distilled water

<u>pH 7.6 buffer</u>

0.2 M Tris 25 ml

0.2 M HCl 19.2 ml

Make up to 100 ml with distilled water